Molecular Biology of the Cell
Vol. 21, 3396–3408, October 1, 2010

Coordination of Substrate Binding and ATP Hydrolysis in Vps4-Mediated ESCRT-III Disassembly

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Submitted June 15, 2010; Revised July 12, 2010; Accepted July 29, 2010
Monitoring Editor: Judith Klumperman

ESCRT-III undergoes dynamic assembly and disassembly to facilitate membrane exvagination processes including multivesicular body (MVB) formation, enveloped virus budding, and membrane abscission during cytokinesis. The AAA-ATPase Vps4 is required for ESCRT-III disassembly, however the coordination of Vps4 ATP hydrolysis with ESCRT-III binding and disassembly is not understood. Vps4 ATP hydrolysis has been proposed to execute ESCRT-III disassembly as either a stable oligomer or an unstable oligomer whose dissociation drives ESCRT-III disassembly. An in vitro ESCRT-III disassembly assay was developed to analyze Vps4 function during this process. The studies presented here support a model in which Vps4 acts as a stable oligomer during ATP hydrolysis and ESCRT-III disassembly. Moreover, Vps4 oligomer binding to ESCRT-III induces coordination of ATP hydrolysis at the level of individual Vps4 subunits. These results suggest that Vps4 functions as a stable oligomer that acts upon individual ESCRT-III subunits to facilitate ESCRT-III disassembly.

INTRODUCTION

Many endosomal cargoes destined for the lysosome are subject to an additional level of sorting during the formation of multivesicular bodies (MVBs) (Slagsvold *et al*., 2006; Piper and Luzio, 2007; Raiborg and Stenmark, 2009). Defects in MVB sorting stabilize cargoes normally targeted for degradation via this pathway and can result in prolonged signaling of activated growth factor receptors that fail to enter the MVB (Gruenberg and Stenmark, 2004; Slagsvold *et al*., 2006; Tanaka *et al*., 2008; Stuffers *et al*., 2009). MVB sorting is conserved throughout eukaryotes, as is the cellular machinery responsible for this reaction: the endosomal sorting complexes required for transport (ESCRTs) and associated proteins (Piper and Katzmann, 2007; Hurley, 2008; reviewed in Davies *et al*., 2009; Raiborg and Stenmark, 2009). ESCRT-0, -I, -II, and -III and associated proteins execute cargo selection and membrane deformation during MVB sorting. The ESCRT machinery also contributes to topologically-similar membrane deformation events including budding of enveloped viruses and membrane abscission during cytokinesis (Morita and Sundquist, 2004; Carlton and Martin-Serrano, 2007; Morita *et al*., 2007; Carlton *et al*., 2008; Lee *et al*., 2008). Thus, ESCRT-mediated membrane deformation impacts cellular physiology at multiple levels.

ESCRT-III is unique among the ESCRTs in that its membrane recruitment and assembly are coincident (Katzmann *et al*., 2001; Babst *et al*., 2002a; Babst *et al*., 2002b; Bilodeau *et al*., 2003). Yeast ESCRT-III comprises four core subunits essential for MVB sorting (Vps20, Snf7, Vps24, and Vps2) and three accessory subunits (Did2, Ist1, and Vps60) that function in a redundant manner (Babst *et al*., 2002a; Dimaano *et al*., 2008; Rue *et al*., 2008; Bajorek *et al*., 2009b; Xiao *et al*., 2009). Although ESCRT-III subunits exhibit a conserved core tertiary structure (Muziol *et al*., 2006; Bajorek *et al*., 2009b; Xiao *et al*., 2009), they display divergence at their carboxyl termini and appear to contribute unique functions to ESCRT-III.

Genetic and biochemical evidence supports a temporal recruitment and assembly of ESCRT-III subunits into a polymer, suggested to take on a fibril form (Babst *et al*., 2002a; Ghazi-Tabatabai *et al*., 2008; Hanson *et al*., 2008; Lata *et al*., 2008; Teis *et al*., 2008; Saksena *et al*., 2009). In vitro addition of core ESCRT-III subunits to giant unilamellar vesicles indicated that ESCRT-III can drive ILV formation; however, continuous ILV formation required the addition of Vps4 to permit repeated ESCRT-III function (Wollert *et al*., 2009). In vivo studies have implicated a role for Vps4 in mediating ESCRT-III disassembly, but the precise roles of Vps4 and ESCRT-III during ILV formation are not understood (Davies *et al*., 2009; Hanson *et al*., 2009). Vps4 is a type 1 AAA-ATPase that contains an amino-terminal Microtubule Interacting and Trafficking (MIT) domain which binds to ESCRT-III (Babst *et al*., 1997; Scott *et al*., 2005a; Scott *et al*., 2005b; Obita *et al*., 2007; Stuchell-Brereton *et al*., 2007; Kieffer *et al*., 2008). The ATPase cycle of Vps4 has been suggested to be impacted by interaction with membrane-associated ESCRT-III polymers: ESCRT-III binding facilitates Vps4 oligomerization; oligomerization stimulates Vps4 ATP hydrolysis;

This article was published online ahead of print in *MBoC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E10–06–0512) on August 4, 2010.

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Table 1. Plasmids and yeast strains

Strains/plasmids	Description/genotype	Reference
MBY52	*vps4Δ:TRP pep4Δ:LEU2 prb1Δ:LEU2*	Katzmann, *et al*., 2003
JPY188	*vps4Δ:TRP pep4Δ:LEU2 vps20Δ:HIS3 vta1Δ:HIS3*	This study
JPY315	*vps4Δ:TRP pep4Δ:LEU2 prb1Δ:LEU2 vps2Δ:HIS3*	This study
JPY296	*vps4Δ:TRP pep4Δ:LEU2 vps24Δ:HIS3*	This study
JPY311	*vps4Δ:TRP pep4Δ:LEU2 prb1Δ:LEU2 did2Δ:HIS3*	This study
JPY309	*vps4Δ:TRP pep4Δ:LEU2 prb1Δ:LEU2 vta1Δ:HIS3*	This study
JPY137	*vps4Δ:TRP pep4Δ:LEU2 vta1:HIS3*	This study
JPY378	*vps4Δ:TRP pep4Δ:LEU2 ist1Δ:HIS3*	This study
JPY310	*vps4Δ:TRP pep4Δ:LEU2 vps60Δ:HIS3*	This study
JPY459	*vps4Δ:TRP pep4Δ:LEU2 vps24Δ:HIS3 did2Δ:HIS3 vta1Δ:HIS3*	This study
JPY373	*vps4Δ:TRP pep4Δ:LEU2 vps20Δ:HIS3 ist1Δ:HIS3*	This study
JPY293	*vps4Δ:TRP pep4Δ:LEU2 vps25Δ:HIS3*	This study
JPY188	*vps4Δ:TRP pep4Δ:LEU2 vps28Δ:HIS3*	This study
JPY140	*vps4Δ:TRP pep4Δ:LEU2 snf7Δ:HIS3*	This study
JPY554	*vps4Δ:TRP pep4Δ:LEU2 snf7Δ:HIS3 vps2Δ:HIS3*	This study
JPY48	*vta1Δ::HIS3*	Azmi, *et al*., 2006
JPY45	*vps4Δ:TRP vta1Δ::HIS3*	Azmi, *et al*., 2006
pMB63	pGEX-4T $Vps4^{E233Q}$	Babst, *et al*., 1998
pMB40	pGEX-4T $Vps4^{\Delta MIT}$	Babst, *et al*., 1998
pMB90	pGEX-4T $Vps4^{\Delta MIT,E233Q}$	Babst, *et al*., 1998
pGST $Vps4^{I18D}$	pGEX-4T $Vps4^{I18D}$	This study
pGST $Vps4^{I18D,E233Q}$	pGEX-4T $Vps4^{I18D}$	This study
pGST $Vps4^{L64D}$	pGEX-4T $Vps4^{L64D}$	This study
pGST $Vps4^{L64D,E233Q}$	pGEX-4T $Vps4^{L64D,E233Q}$	This study
pMB168	pRS416 Vps20-HA	Babst, *et al*., 2002a
pET28Vta1	pET28b Vta1	Azmi, *et al*., 2006
pMB106	pRS416 Vps24-GFP	This study
pCherryDid2	pRS415 (Vps21 Prom.) Cherry-Did2	This study
pMB97	pRS426 $Vps4^{E233Q}$-GFP	This study
pMB341	pRS416 GFP-$Vps4^{\Delta MIT,E233Q}$	This study
p416 $ChVps4^{E233Q}$	pRS415 (Vps21 Prom.) Cherry-$Vps4^{E233Q}$ 1–437	This study
p416 $ChVps4^{\Delta MIT,E233Q}$	pRS415 (Vps21 Prom.) Cherry-$Vps4^{E233Q}$ 118–437	This study
pMB118	pRS425 GFP-CPS	This study
pRS426 Snf7	pRS426 (Snf7 Prom.) Snf7 1–240	This study
pRS426 $Snf7^{Vps2MIM1}$	pRS426 (Snf7 Prom.) Snf7 1–227::Vps2 218–232	This study

and ATP hydrolysis has been proposed to lead to both Vps4 oligomer dissociation and ESCRT-III disassembly, releasing ESCRT-III subunits into the cytoplasm (Babst *et al*., 1997; Babst *et al*., 1998; Scott *et al*., 2005a; Landsberg *et al*., 2009). However, an alternative model has proposed that Vps4 functions as a stable oligomer during ESCRT-III disassembly, as suggested by the oligomerization-promoting activity of the Vps4 cofactor Vta1, by mutation of the Vps4 central pore, and by analogy to other AAA-ATPases (Scott *et al*., 2005a; Azmi *et al*., 2006; White and Lauring, 2007; Gonciarz *et al*., 2008; Yu *et al*., 2008; Landsberg *et al*., 2009). While Vps4 disassembly of ESCRT-III plays a critical role in MVB sorting, the mechanism by which Vps4 disassembles ESCRT-III and the means by which ESCRT-III assembly and Vps4-mediated disassembly are coordinated are unclear.

To understand the role of Vps4 in ESCRT-III disassembly, an in vitro system was developed for characterizing ESCRT-III and Vps4 contributions. This system recapitulated observations of experiments in yeast, validating its use for examining Vps4 disassembly of ESCRT-III. Our analyses revealed that concerted hydrolysis is not required within the Vps4 oligomer and that not all subunits need to engage ESCRT-III via MIT domains at the same time. However, incorporation of full-length Vps4 subunits incapable of hydrolyzing ATP ($Vps4^{E233Q}$) disrupted Vps4-mediated ESCRT-III disassembly, uncovering coordination between ESCRT-III binding via the MIT domain and ATP hydrolysis at the subunit level within the Vps4 oligomer. These results further suggest that Vps4 can function as a stable oligomer acting upon individual ESCRT-III subunits to facilitate ESCRT-III polymer disassembly in a processive manner.

MATERIALS AND METHODS

Plasmid Construction and Yeast Strains

Standard molecular biology and yeast genetics were used to generate plasmids and strains as listed in Table 1. Mutation of *VPS4* was performed using the GeneTailor Site Directed Mutagenesis System (Invitrogen, Carlsbad, CA). All plasmids were sequenced to identify and exclude unanticipated mutations.

Protein Expression and Purification

Expression and purification of Vps4, Vps4 mutants, and Vta1 were performed as previously described (Babst *et al*., 1998; Azmi *et al*., 2006). Briefly, a short induction (1–2 h) with 500 μM isopropyl-β-D-1-thiogalactopyranoside IPTG was used to express GST-Vps4 fusions, which were subsequently affinity purified with Glutathione Sepharose 4B (GE Healthcare, Piscataway, NJ). Induction of His_6-Vta1 was conducted overnight at 22°C, and the protein was purified by Ni^{+2} affinity chromatography. After elution, the GST and His_6 epitopes were removed by treatment with Thrombin, and the proteins were purified using a BioSource Q2 anion exchange column (Bio-Rad, Hercules, CA).

ATPase Assay

WT and mutant Vps4 ATPase activities were analyzed by ATPase assays as previously described (Babst *et al*., 1998). All reactions were initiated with 1 mM ATP, and quantitation of activity was performed with time-points exhibiting <30% ATP hydrolysis (to avoid complications from product inhibition). When mixings of wild-type and mutant Vps4 were performed in conjunction with the ATPase assay, proteins were preincubated for at least 10

min, and the activity was assessed as ADP generated per wild-type Vps4 per minute. For Figures 4C and 6B, 250 nM Vps4 was used to permit molar excesses of $Vps4^{E233Q}$ or $Vps4^{\Delta MIT,E233Q}$ with lesser amounts of these proteins. For the dilution analysis depicted in Supplemental Figure 3, C and D, standard 20-μl reactions were initiated. After removal of the first three time points to evaluate initial activities, reactions were diluted by addition of nine volumes of 1× ATPase buffer with 1 mM ATP at 10 min after initiation of the reaction. ATP to ADP conversion was then evaluated at up to 260 min post dilution. The ADP generated per Vps4 was determined and graphed versus time. Linear regression was used at assess ATPase activities before and after dilution (Prizm, Graphpad Software, La Jolla, CA). A similar procedure was used for the dilution analysis depicted in Figure 7, A and B, except dilution was initiated at 30 min and included or excluded additional ATP during dilution. Readdition of ATP to 1 mM (to the samples diluted without additional ATP) was performed 30 min after dilution. The reactions were performed in triplicate and the experiment performed twice. ADP/Vps4 amounts were determined at 5-min intervals, and linear regression was used to assess ATPase activities. The initial rate of hydrolysis (I) was determined using the 5- to 15-min time points (before 30% ATP hydrolysis). The rate of activity following dilution with ATP (II) was assessed using the 35- to 90-min time points (blue). The rate of activity after dilution without maintaining ATP at 1 mM (III) was assessed using the 35- to 90-min time points (black). The rate of activity after dilution and with ATP readdition to 1 mM (IV) was assessed using the 65- to 90-min time points (red). Activities relative to the initial rate (I) are presented. 500 nM Vps4 was used in these dilution experiments as it is a concentration below the apparent K_m, the ATPase activities of 50 nM and 500 nM Vps4 are readily detectable and distinct, these concentrations do not require excessive amounts of Vps4, and adequate time-points can be taken before reaching conditions with apparent product inhibition.

In Vitro ESCRT-III Release Assay

Membrane Generation. One liter of yeast cells were grown at 30°C in Yeast Extract Peptone Dextrose medium (YPD) to an optical density at 600 nm (OD_{600}) of 0.5–0.8. Cells were harvested, softened, and spheroplasted as previously described (Vida *et al*., 1990). Spheroplasted cells were harvested by centrifugation at 3,000*g* for 5 min and resuspended in chilled lysis buffer [0.2M Sorbitol, 50 mM KOAc, 2 mM EDTA, 20 mM HEPES, pH 6.8 with complete protease inhibitors (Roche)] and lysed by 15 strokes with a glass tissue homogenizer. Intact cells were removed by 500*g* centrifugation for 5 min. The 500*g* supernatant was carefully removed and centrifuged at 13,000*g* for 10 min to generate a pellet (P13) fraction. The P13 was resuspended in 1 ml ATPase reaction buffer (100 mM KOAc, 20 mM HEPES pH 7.4, 5 mM MgOAc) with 1 mM sorbitol and protease inhibitors (Buffer A), and the 13,000*g* spin was repeated to remove contaminating soluble material. The repelleted P13 fraction was resuspended in Buffer A and passed through an 18-gauge needle three times and a 30-gauge needle five times to homogenize the resuspended membranes. Membranes were diluted to 50 OD_{600}/ml in buffer A and stored in −80°C until further use.

ESCRT-III Disassembly Assay. Twenty-microliter reactions were set up with 0.5 OD_{600} equivalent of resuspended P13 membranes. This amount of membranes is anticipated to yield an Snf7/ESCRT-III concentration in the low nM range (~1–3 nM), as assessed both by Western blotting with dilutions of purified Snf7 (data not shown) and by calculations based on the number of molecules of Snf7 per cell (Ghaemmaghami *et al*., 2003). The ATP regeneration system (10 mM phosphocreatine, 10 U/ml creatine phosphokinase, and 1 mM ATP) and purified Vps4 were added in the form of drops on the side of the tube to prevent mixing before reaction initiation. The reaction was initiated by a brief spin (~5 s at 4°) and was incubated at 30°C in a rotator for 10 min. The reaction was terminated by centrifugation at 13,000*g* for 10 min at 4°C. The soluble fraction from the 13,000*g* spin (S13) was combined with 5 μL of 5× sample buffer, and the P13 was resuspended in 25 μL of 5× sample buffer. Five microliters of the reaction was run on a 15% gel, and Western blotting was performed with anti-Snf7 polyclonal antibody (1:10,000) (Babst *et al*., 1998). Reactions were performed in triplicate within an experiment, and experiments were performed at least thrice. Error bars indicate the SE of the mean. The use of 100 nM as the standard Vps4 concentration in the ESCRT-III disassembly assay was empirically determined as the concentration providing 50% Snf7 release from the membrane in reactions with 0.5 OD_{600} equivalents of membrane incubated for 10 min at 30°.

Biochemical Analyses. Immunoprecipitation (IP) experiments were performed as described (Babst *et al*., 2002a) using ESCRT-III release material. Briefly, ESCRT-III release assays were performed as above except additional substrate (10 OD_{600} equivalents), Vps4 (500 nM) and time (30 min) were used. Total reactions (for Snf7-Vps20HA coimmunoprecipitations, as Vps20HA release from the membrane was complicated by its myristolation) or the pelleted material (for Snf7-Vps24 coimmunoprecipitations) were then extracted with 1% TritonX-100 for 10 min on ice. Samples were diluted 10-fold, and the insoluble material was removed by centrifugation. Immunoprecipitation with either Snf7 or Vps24 antisera was performed, and antibody complexes were isolated with Protein A Sepharose. Isolated material and input fractions were then analyzed by Western blotting with HA.11 mAb (Covance, Princeton, NJ) or polyclonal antisera against Snf7 (Babst *et al*., 1998). Quantitation of Vps20HA (Figure 1D) represents the mean from three independent experiments. The immunoprecipitation of Snf7 under native conditions was more efficient after release from the membrane (Figure 1C), suggesting that epitopes recognized by the Snf7 antisera are occluded in the ESCRT-III assembled state.

Subcellular Localization

Microscopy was performed on living cells using the DeltaVisionRT System (Applied Precision, Issaquah, WA) including a fluorescence microscope (Nikon) equipped with EGFP and Cherry filter sets and a digital camera (Coolsnap HQ, Photottmetrix). Images were processed using the DeltaVision software package (Applied Precision). The Cherry-Did2 fusion used for colocalization is a partially functional form of Did2 (data not shown). Strains lacking the Vps4 cofactor Vta1 (*vta1*Δ and *vta1*Δ *vps4*Δ) were used in the colocalization study to avoid complications from Vta1-dependent recruitment to the endosome (Shestakova *et al*., 2010) as well as to enhance Cherry-Did2 endosomal localization (Nickerson *et al*., 2010). Cherry-Did2 colocalized with Vps24-GFP in *vps4*Δ cells (Figure 5A), confirming the Cherry-Did2 compartment as an ESCRT-III-positive endosome.

RESULTS

Reconstitution of Vps4-Dependent ESCRT-III Disassembly and Membrane Release

Two general models for Vps4 function have been proposed: 1) Vps4 undergoes a dynamic cycle of oligomerization and dissociation to facilitate ESCRT-III disassembly in mass; and 2) Vps4 functions as a stable oligomer disassembling the ESCRT-III polymer. To explore the mechanism of Vps4 function, an in vitro assay was developed that reconstituted Vps4-mediated ESCRT-III membrane release. This assay monitored the redistribution of ESCRT-III subunits from membrane into soluble fractions.

To validate the assay, the Vps4-, energy-, and time-dependences of ESCRT-III subunit (Snf7 or Vps24) release from the membrane fraction were examined. Ten-minute incubation of ESCRT-III-containing membranes at 30°C with 100 nM Vps4, 1 mM ATP, and an ATP regeneration system resulted in a 50% decrease in membrane-associated Snf7 (P13) with a commensurate increase of Snf7 in the soluble fraction (S13) (Figure 1, A and B). Snf7 release into the S13 was dependent on both ATP and Vps4 (Figure 1, A and B) as was Vps24 (Supplemental Figure 1A). Consistent with in vivo studies (Babst *et al*., 1998), the catalytically inactive $Vps4^{E233Q}$ failed to support Snf7 release into the S13 fraction (Figure 1, A and B). Time dependence and Vps4-concentration dependence of Snf7 release were also observed (Supplemental Figure 1B). Ten-minute incubation at 30°C with 100 nM Vps4 and 1 mM ATP was selected as standard reaction conditions as these parameters generated ~50% Snf7 release from the membrane fraction.

To address whether ESCRT-III release from the membrane corresponded to disassembly of the ESCRT-III polymer, the association of the ESCRT-III subunits Vps20 and Snf7 was examined after the release reaction. Vps20HA-containing membranes were incubated with ATP alone or with Vps4 and ATP, and Vps20HA association with Snf7 was assessed by coimmunoprecipitation from detergent-solubilized reactions. Vps4 reduced the association of Vps20HA with Snf7 (Figure 1, C and D) commensurate with Snf7 release from the membrane fraction (Figure 1E). Similarly, Snf7 coimmunoprecipitation with Vps24 was abrogated upon ESCRT-III release from the membrane (Supplemental Figure 1C). These results demonstrate that the ESCRT-III membrane release is coincident with disassembly of ESCRT-III. As such, this in vitro assay will be referred to as the ESCRT-III disassembly assay or the ESCRT-III membrane release assay interchange-

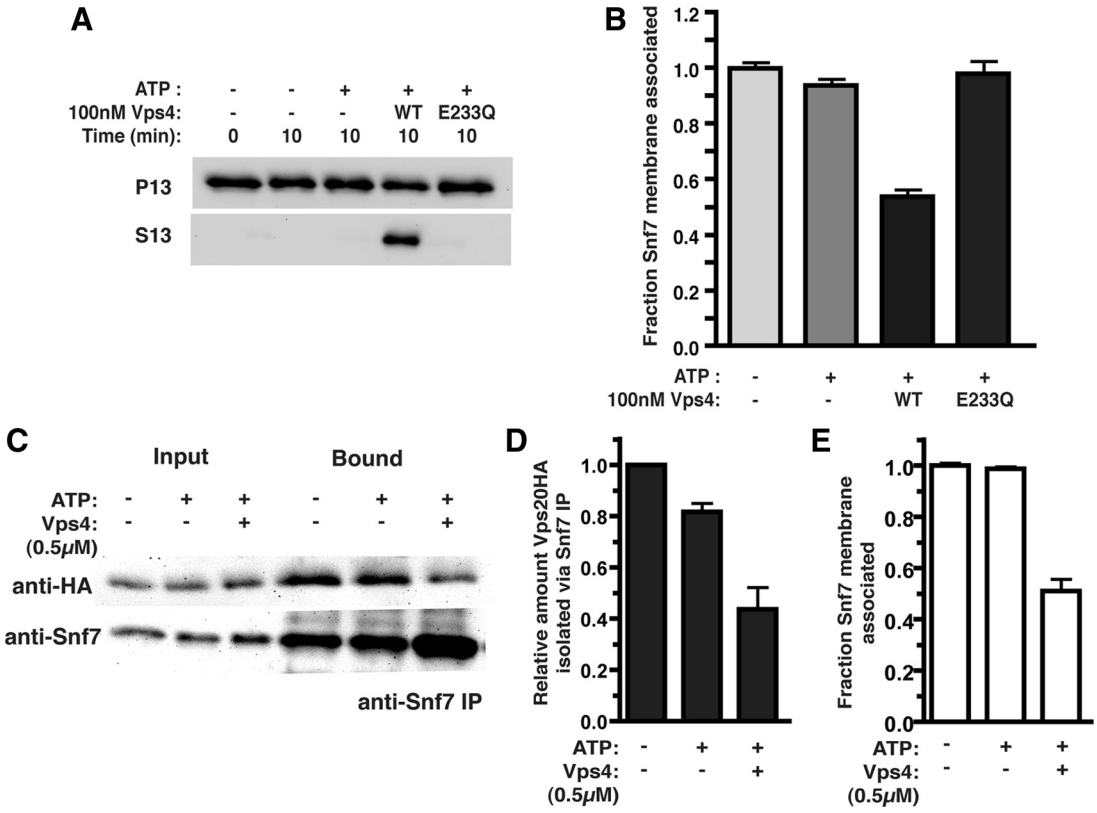

Figure 1. In vitro ESCRT-III release and disassembly. (A) ESCRT-III release reactions were initiated with resuspended P13 membranes (0.5 OD_{600} equivalents) generated from *vps4*Δ cells, in the presence (+) or absence (−) of 1 mM ATP and an ATP regeneration system (ATP), 100 nM Vps4 (WT), or 100 nM $Vps4^{E233Q}$ (E233Q). After a 10 min incubation at 30°C, the membrane-associated (P13) and soluble (S13) materials were separated.Western blotting with Snf7 antiserum was performed. (B) Quantitation of ESCRT-III release reaction. Data are presented as the fraction of Snf7 remaining membrane associated and represent the mean of at least three independent experiments with reactions performed in triplicate within each experiment. (C) Vps20HA-containing membranes (10 OD_{600} equivalents) were incubated for 30 min with 500 nM Vps4 and ATP or ATP alone. Snf7 immunoprecipitations were then performed after TritonX-100 extraction of the total reaction. Vps20HA was detected with the HA.11 mAb and Snf7 with the polyclonal antiserum. (D) Quantitation of Vps20HA isolation from the Snf7 immunoprecipitation. (E) Quantitation of the fraction of Snf7 remaining membrane associated under the reaction conditions used in C.

ably. These results demonstrate that Vps4 disassembles the ESCRT-III polymer, as suggested by observations correlating ESCRT-III assembly state with Vps4 function (Babst *et al*., 1998; Babst *et al*., 2002a).

In vitro ESCRT-III Disassembly Depends on Did2, Vps24, and Vps2

Vps4 disassembly of ESCRT-III appears to be impacted by the ESCRT-III subunit composition as yeast lacking Vps24, Vps2, or Did2 exhibit stabilized ESCRT-III membrane association (Babst *et al*., 2002a; Nickerson *et al*., 2006; Teis *et al*., 2008). To examine these ESCRT-III subunit contributions, Vps4 ESCRT-III disassembly reactions were performed with membranes isolated from yeast lacking Vps4 and individual ESCRT-III subunits (i.e., *vps4*Δ *vps60*Δ). Membrane fractions from each strain contained Snf7; however, loss of Vps20 partially compromised Snf7 abundance (Supplemental Figure 2A). Losses of the ESCRT-III subunits Vps60, Ist1, or Vps20 or of ESCRT-I (Vps28) or ESCRT–II (Vps25) subunits did not perturb Vps4-mediated Snf7 membrane release (Figure 2, A and B, and data not shown). By contrast, losses of Vps24, Vps2, or Did2 abrogated Snf7 membrane release catalyzed by 100 nM Vps4 (Figure 2, A and B). However, increased Vps4 concentration (1 μM Vps4) partially restored ESCRT-III disassembly in the absences of Vps24 or Did2 but not in the absence of Vps2 (Figure 2, A and B) or in the combined absence of Vps24 and Did2 (Supplemental Figure 2B). These results implicated distinct roles for Did2, Vps24, and Vps2 in promoting Vps4 disassembly of ESCRT-III. However, we cannot distinguish whether these contributions are related to assembly of ESCRT-III polymers or as participants in the disassembly reaction. Regardless, Vps2 contributes to Vps4 disassembly of ESCRT-III to a more significant extent while Vps24 and Did2 make partially redundant contributions.

Vps2, Vps24, and Did2 contain a carboxyl-terminal sequence motif (MIT-interacting motif 1, MIM1) that mediates association with Vps4 (Obita *et al*., 2007; Stuchell-Brereton *et al*., 2007). The distinct contributions of Vps2, Vps24, and Did2 suggested their MIM1 elements are not functionally equivalent and that the Vps2 MIM1 contributes a critical function in ESCRT-III disassembly. To evaluate this contribution, a chimeric ESCRT-III subunit ($Snf7^{Vps2MIM1}$) was generated by replacing the Snf7 α6 with the Vps2 α6 and its MIM1 (Snf7 1–227 fused to Vps2 218–232). Snf7 or $Snf7^{Vps2MIM1}$ were expressed in *vps4*Δ *snf7*Δ or *vps4*Δ *snf7*Δ *vps2*Δ yeast, and membranes generated from these strains were analyzed for ESCRT-III disassembly by Vps4. Whereas loss of Vps2 abrogated Vps4 release of Snf7 from these membranes, release of $Snf7^{Vps2MIM1}$ occurred independent of Vps2 (Figure 2C). This result implicated the Vps2 MIM1 as playing an important role in ESCRT-III disassembly by

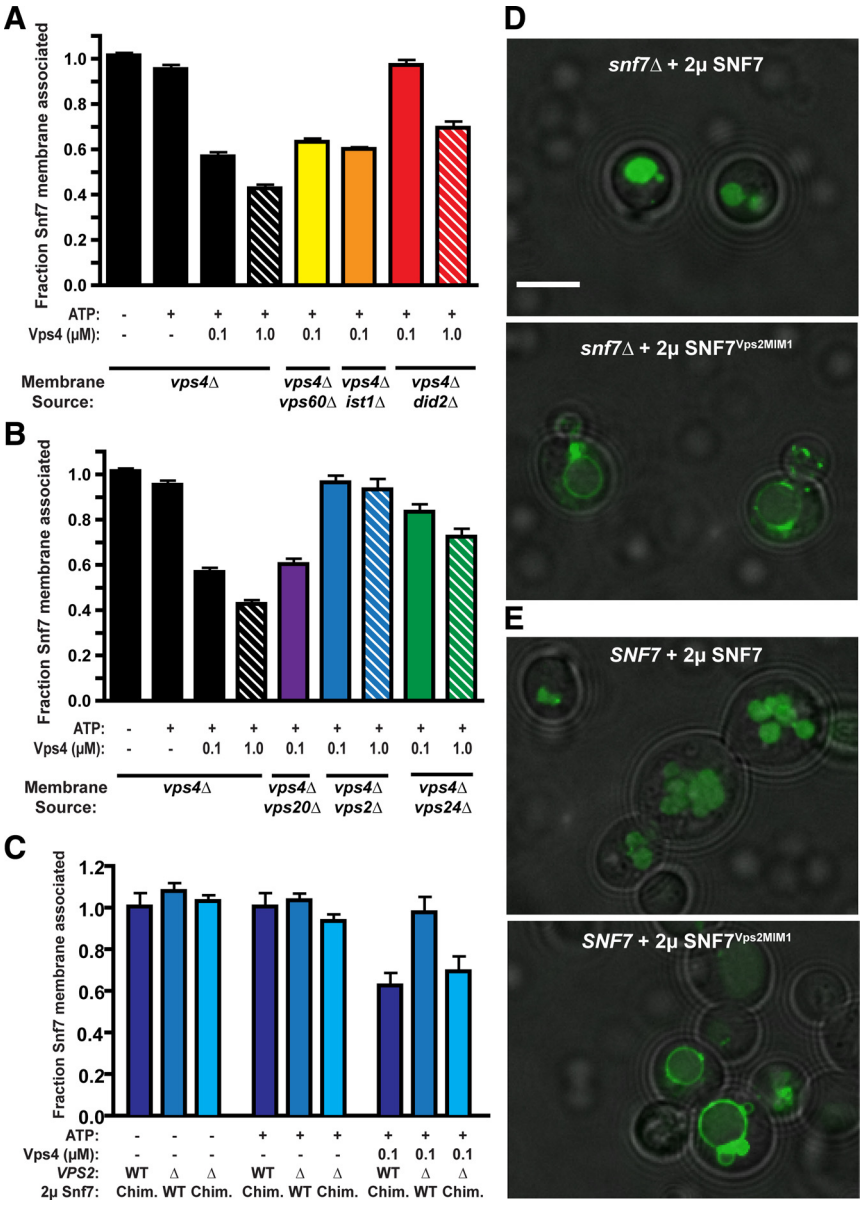

Figure 2. ESCRT-III subunit contributions to Vps4 disassembly of ESCRT-III. (A) ESCRT-III–containing membranes were generated from yeast lacking the accessory ESCRT-III subunits Did2 (red), Ist1 (orange), and Vps60 (red). Membranes (0.5 OD_{600} equivalent) were incubated with ATP and 100 nM or 1 μM Vps4, and the fraction of Snf7 remaining membrane associated was determined. (B) ESCRT-III membrane release assays performed with membranes generated from yeast lacking the core ESCRT-III subunits Vps20 (purple), Vps24 (green), and Vps2 (blue). (C) ESCRT-III membrane release assays performed with membranes generated from yeast lacking Snf7 and Vps2 (Δ) or Snf7 alone (WT) and expressing either Snf7 or the $Snf7^{Vps2MIM1}$ chimera. (D and E) Distribution of the MVB sorting reporter GFP-CPS in *snf7*Δ (D) or wild-type yeast (E) expressing Snf7 or the $Snf7^{Vps2MIM1}$ chimera. Bar, 5 μm.

Vps4 and suggests that the remainder of Vps2 is dispensable. To extend this analysis, the localization of the MVB cargo GFP-Carboxypeptidase S (GFP-CPS) was examined in *snf7*Δ yeast expressing Snf7 or $Snf7^{Vps2MIM1}$. Whereas Snf7 reexpression restored GFP-CPS localization to the lumen of the vacuole, the $Snf7^{Vps2MIM1}$ chimera failed to complement the *snf7*Δ MVB sorting defects, as GFP-CPS localized to the vacuolar limiting membrane (Figure 2D). Moreover, $Snf7^{Vps2MIM1}$ disrupted GFP-CPS localization to the vacuolar lumen of wild type cells (*SNF7*), demonstrating a dominant negative effect on MVB sorting (Figure 2E). These results indicated that while the Vps2 MIM1 element can bypass the requirement for Vps2 in Vps4-mediated Snf7 membrane release, the $Snf7^{Vps2MIM1}$ chimera does not support MVB sorting.

ESCRT-III Disassembly Is Dependent on Vps4 MIM1 and MIM2 Interactions

Vps4 interacts with ESCRT-III subunits via its amino-terminal MIT domain (Obita *et al.*, 2007; Stuchell-Brereton *et al.*, 2007; Kieffer *et al.*, 2008). Although ATPase activity is not affected (Supplemental Figure 3A and Babst *et al.*, 1998; Azmi *et al.*, 2006), deletion of this domain ($Vps4^{\Delta MIT}$) abrogates MVB sorting (Vajjhala *et al.*, 2007). Similarly, $Vps4^{\Delta MIT}$ failed to support ESCRT-III disassembly (Figure 3), indicating that the MIT domain is required for ESCRT-III disassembly and that Vps4 ATP hydrolysis is not sufficient for this activity. Vps4 mutants predicted to be defective for either MIM2 interaction ($Vps4^{I18D}$) or MIM1 interaction preferentially ($Vps4^{L64D}$) (Supplemental Figure 3, A and B and Kieffer *et al.*, 2008) were also analyzed to examine contributions of these two modes of MIT-ESCRT-III association. $Vps4^{L64D}$ was incapable of supporting ESCRT-III disassembly (Figure 3), consistent with Vps4 ESCRT-III disassembly assay dependence on Vps2, Vps24, and Did2 (Figure 2, A and B). While 100 nM $Vps4^{I18D}$ did not support Snf7 release from the membrane, 1 μM $Vps4^{I18D}$ exhibited ESCRT-III disassembly activity (Figure 3). The inability of 100 nM $Vps4^{I18D}$ to support disassembly contrasts with the findings that this reaction was not perturbed by individual or combined losses

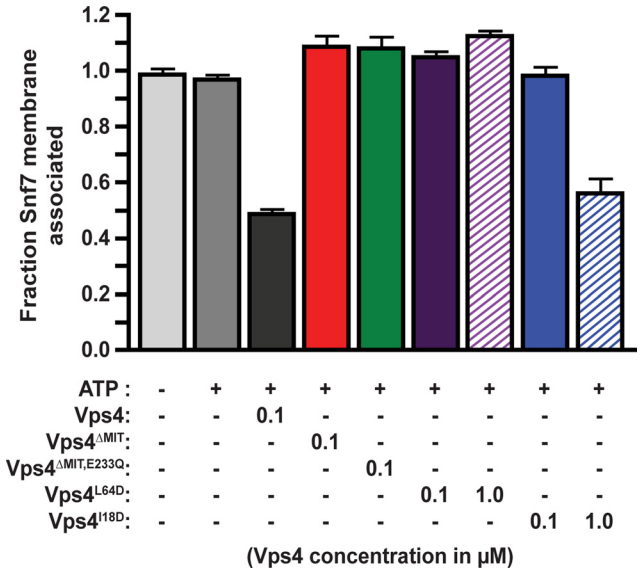

Figure 3. ESCRT-III disassembly depends on Vps4 MIT associations with MIM1 and MIM2. ESCRT-III disassembly reactions were performed with inclusion of 100 nM Vps4 (black), 100 nM Vps4$^{\Delta MIT}$ (red), 100 nM Vps4$^{\Delta MIT,E233Q}$ (green), 100 nM and 1 μM Vps4^{L64D} (purple, MIM1 binding surface), or 100 nM and 1 μM Vps4^{I18D} (blue, MIM2 binding surface).

of MIM2-containing ESCRT-III subunits Vps20 and Ist1 (Figure 2, A and B, and data not shown), suggesting that ESCRT-III subunit redundancy may exist for the MIM2-Vps4 interactions. These results also indicated that Vps4 MIT interactions via both MIM1 and MIM2 binding surfaces contribute to ESCRT-III disassembly.

Coordination within the Vps4 Oligomer During ESCRT-III Disassembly

Vps4 functions as an oligomer, likely comprising 12 subunits (Hanson and Whiteheart, 2005; White and Lauring, 2007; Yu *et al*., 2008; Landsberg *et al*., 2009). To examine the relationships among Vps4 subunits, their ATPase activity and engagement with ESCRT-III within the oligomer, ESCRT-III disassembly assays were performed with mixtures of wild-type and mutant Vps4 subunits.

To test whether a full complement of MIT domains is required within the Vps4 oligomer, Vps4$^{\Delta MIT}$ subunits were mixed with wild-type subunits (Vps4) in the ESCRT-III disassembly assay. Mixing of Vps4 (100 nM) with Vps4$^{\Delta MIT}$ (100 nM, 500 nM, and 1 μM) generated disassembly activities indistinguishable from Vps4 alone (Figure 4A). Similar results were observed upon mixing Vps4$^{\Delta MIT,E233Q}$ subunits with Vps4 (Figure 4B). These results suggested that the Vps4 oligomer does not require a full complement of MIT domains to be functional.

However, an alternative explanation was that Vps4$^{\Delta MIT}$ and Vps4$^{\Delta MIT,E233Q}$ subunits were not incorporated into Vps4 oligomers in this assay. To address this possibility, the ability of Vps4$^{\Delta MIT,E233Q}$ to promote ATPase activity of Vps4 subunits was examined. Vps4 ATP hydrolysis exhibits concentration-dependent specific activity, attributed to the role of oligomerization in Vps4 ATP hydrolysis (Babst *et al*., 1998; Azmi *et al*., 2006). While Vps4$^{\Delta MIT,E233Q}$ did not exhibit ATPase activity by itself (Supplemental Figure 3A), addition of 1 or 2 μM Vps4$^{\Delta MIT,E233Q}$ to 250 nM Vps4 enhanced the ATPase activity of the Vps4 subunits (Figure 4C), consistent with formation of Vps4-Vps4$^{\Delta MIT,E233Q}$ hetero-oligomers in vitro. To further address this issue, the ESCRT-III disassembly assay was performed with 30 nM Vps4 in the presence of excess Vps4$^{\Delta MIT,E233Q}$ (1.2 μM). The addition of 1.2 μM Vps4$^{\Delta MIT,E233Q}$ significantly enhanced ESCRT-III disassembly mediated by 30 nM Vps4 (Figure 4D). This result supports the model that Vps4$^{\Delta MIT,E233Q}$ forms hetero-oligomers with Vps4 in vitro that support ESCRT-III disassembly. To address whether hetero-oligomer formation occurs in vivo, the association of GFP-Vps4$^{\Delta MIT,E233Q}$ with ESCRT-III-positive endosomes was examined in yeast lacking Vta1 (see Materials and Methods). Vps4^{E233Q}-GFP colocalized with Cherry-Did2 (Figure 5B), indicating Vps4^{E233Q}-GFP recruitment to ESCRT-III–positive endosomes. GFP-Vps4$^{\Delta MIT,E233Q}$ exhibited partial colocalization with Did2-positive endosomes in *vta1*Δ yeast (Figure 5D, Supplemental Figure 4A), but this association was no longer evident in *vta1*Δ *vps4*Δ cells (Figure 5C, Supplemental Figure 4B). The Vps4-dependence of GFP-Vps4$^{\Delta MIT,E233Q}$ localization to ESCRT-III–positive endosomes supported the assertion that Vps4$^{\Delta MIT,E233Q}$ forms hetero-oligomers with Vps4, consistent with recent observations (Shestakova *et al*., 2010). These analyses suggested that the Vps4-Vps4$^{\Delta MIT,E233Q}$ hetero-oligomer forms both in vivo and in vitro and supported the conclusion that the Vps4 oligomer does not require a full complement of MIT domains to support ESCRT-III disassembly.

To assess the contribution of Vps4 subunit ATPase activity in ESCRT-III disassembly, mixing experiments were performed with wild-type and Vps4^{E233Q} subunits. Addition of equal molar Vps4^{E233Q} inhibited 100 nM Vps4 ESCRT-III disassembly (Figure 6A). Moreover, reduction of the molar ratio to 1:2 (Vps4^{E233Q}:Vps4) also inhibited Snf7 membrane release. These results suggested that incorporation of subunits that cannot hydrolyze ATP disrupted Vps4 oligomer activity. However, addition of 1 μM Vps4$^{\Delta MIT,E233Q}$ to 100 nM Vps4 (10:1) failed to inhibit ESCRT-III disassembly (Figure 4B). This contrast indicated that Vps4^{E233Q} inhibition of ESCRT-III disassembly was dependent on the MIT domain (Figure 8). The inability of Vps4$^{\Delta MIT,E233Q}$ subunits to inhibit the Vps4-Vps4$^{\Delta MIT,E233Q}$ hetero-oligomer also indicated that every subunit of the Vps4 oligomer does not require catalytic activity to permit ESCRT-III disassembly. However, the inhibitory activity of Vps4^{E233Q} suggested that when a subunit engages ESCRT-III via its MIT domain, ATP hydrolysis by that subunit is required. These observations refine the understanding of subunit composition and action of the Vps4 oligomer by uncovering an intimate link between the engagement of ESCRT-III via the MIT domain and ATP hydrolysis by the engaged Vps4 subunit during ESCRT-III disassembly.

To further explore Vps4^{E233Q} inhibitory activity, Vps4^{E233Q} effects on ATPase activity of Vps4 subunits was examined. Vps4^{E233Q} addition enhanced the ATPase activity of 250 nM Vps4 in a concentration-dependent manner (Figure 6B). Furthermore, titration of Vps4 subunits in the presence of 2 or 4 μM Vps4^{E233Q} indicated that Vps4^{E233Q} addition reduced the Vps4 apparent K_m while the Vps4 V_{max} was not enhanced (Figure 6C). These effects are consistent with Vps4^{E233Q} supporting hetero-oligomer formation without disrupting wild-type subunit ATPase activity, as observed with Vps4$^{\Delta MIT,E233Q}$-Vps4 mixtures (Figure 4C). The dissimilar effects of Vps4^{E233Q} and Vps4$^{\Delta MIT,E233Q}$ subunits on Vps4 disassembly of ESCRT-III (Figures 4B and 6A) suggested that the presence of ESCRT-III itself plays a critical role in MIT-dependent Vps4^{E233Q} inhibitory activity.

To examine the role of the MIT domain in Vps4^{E233Q} inhibition in vivo, GFP-CPS localization was examined

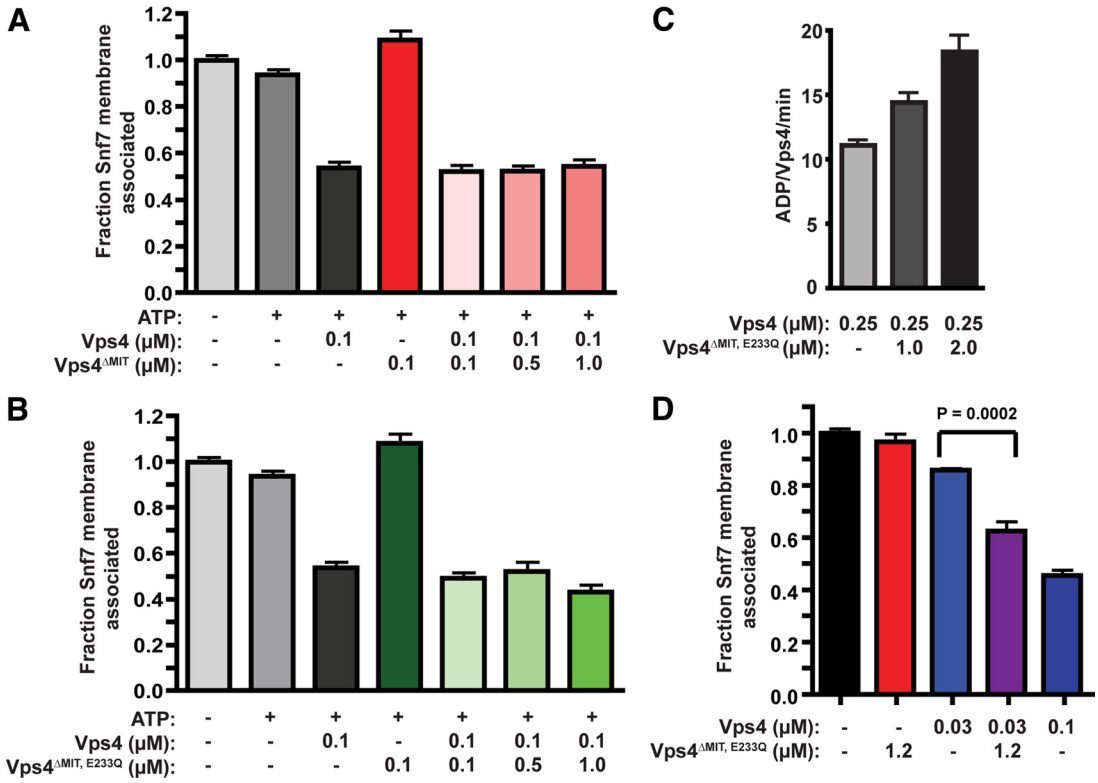

Figure 4. Vps4 oligomer MIT domain composition for ESCRT-III disassembly. (A) ESCRT-III disassembly reactions were performed with *vps4*Δ membranes (0.5 OD_{600} equivalents) and 100 nM Vps4, 100 nM $Vps4^{\Delta MIT}$, or 100 nM Vps4 with increasing concentrations of $Vps4^{\Delta MIT}$ (100 nM, 500 nM, and 1 μM). (B) ESCRT-III release reactions were performed as in A but with increasing concentrations of $Vps4^{\Delta MIT,E233Q}$ (100 nM, 500 nM, and 1 μM). (C) ATPase activity of 250 nM Vps4 alone or with addition of 1 μM or 2 μM $Vps4^{\Delta MIT,E233Q}$. The initial ATP concentration was 1 mM. Activity is presented as ADP generated per wild-type Vps4 per minute. (D) ESCRT-III disassembly reactions were performed as in A but with 30 nM Vps4 alone, 1.2 μM $Vps4^{\Delta MIT,E233Q}$ alone, 30 nM Vps4 and 1.2 μM $Vps4^{\Delta MIT,E233Q}$, or 100 nM Vps4. The addition of $Vps4^{\Delta MIT,E233Q}$ enhanced 30 nM Vps4-mediated ESCRT-III disassembly ($p < 0.0002$).

in yeast expressing $Vps4^{E233Q}$ or $Vps4^{\Delta MIT,E233Q}$. Cherry-$Vps4^{E233Q}$ expression resulted in GFP-CPS delivery to the limiting vacuolar membrane, indicating defective MVB sorting (Figure 6D). By contrast, GFP-CPS localization to the vacuolar lumen was not perturbed by Cherry-$Vps4^{\Delta MIT,E233Q}$ expression (Figure 6E), indicating that the $Vps4^{E233Q}$ dominant negative effect on MVB sorting is dependent on the MIT domain. This conclusion is consistent with recently reported observations (Shestakova *et al*., 2010) and is also supported by the largely diffuse distribution, with only limited punctate localization, of Cherry-Did2 upon GFP-$Vps4^{\Delta MIT,E233Q}$ expression in *vta1*Δ cells (Figure 5D). This Did2 pattern is similar to the distribution in *vta1*Δ cells and is distinct from the extensive Cherry-Did2 endosomal accumulation in *vps4*Δ cells or in cells expressing $Vps4^{E233Q}$-GFP (Figure 5A and data not shown). These observations support the conclusion that the $Vps4^{E233Q}$ dominant negative effect on MVB sorting is dependent on the MIT domain, consistent with observations using the ESCRT-III disassembly assay.

Vps4 Functions as a Stable Oligomer In Vitro

Vps4 dissociation upon ATP hydrolysis has been suggested to contribute to the disassembly of ESCRT-III (Babst *et al*., 1998; Landsberg *et al*., 2009); however, Vps4 has also been suggested to function as a stable AAA-ATPase oligomer (Scott *et al*., 2005a; Gonciarz *et al*., 2008). Because the stability of the Vps4 oligomer is relevant for the mechanism of ESCRT-III disassembly, it was important to determine whether dissociation of the Vps4 oligomer is an implicit step within the ATP hydrolysis cycle.

Reaction conditions of the ATP hydrolysis assay were modified to test Vps4 functional stability. Vps4 exhibits concentration dependent activity (Figure 6C and Babst *et al*., 1998; Azmi *et al*., 2006). If dissociation of the Vps4 oligomer is implicit within the ATP hydrolysis cycle, then Vps4 ATPase activity should be sensitive to dilution. However, if the Vps4 oligomer is stable through multiple rounds of ATP hydrolysis, then ATPase activity should be resistant to dilution once the Vps4 oligomer forms. ATPase reactions were initiated with 500 nM Vps4 in the presence of 1 mM ATP. After 30 min, reactions were diluted 10-fold (to 50 nM Vps4) and the continued hydrolysis of ATP was assessed (Figure 7A). Dilution of Vps4 10-fold in the presence of 1 mM ATP resulted in ATPase activity equivalent to the initial rate (Figure 7B), and this activity was undiminished over an additional 60 min. However, if Vps4 was diluted without maintaining the ATP level and then ATP was subsequently restored to 1 mM, the resulting specific activity was reduced by 78% (Figure 7B). This analysis suggested that the Vps4 oligomer dissociates when ATP becomes limiting but is functionally stable when ATP is present. Furthermore, similar dilution experiments revealed that the functional stability of Vps4 ATPase activity is maintained for more than 4 h post dilution in the presence of ATP (Supplemental Figure 3, C and D). These observations support the conclusion that Vps4 can function as a stable oligomer rather than a model

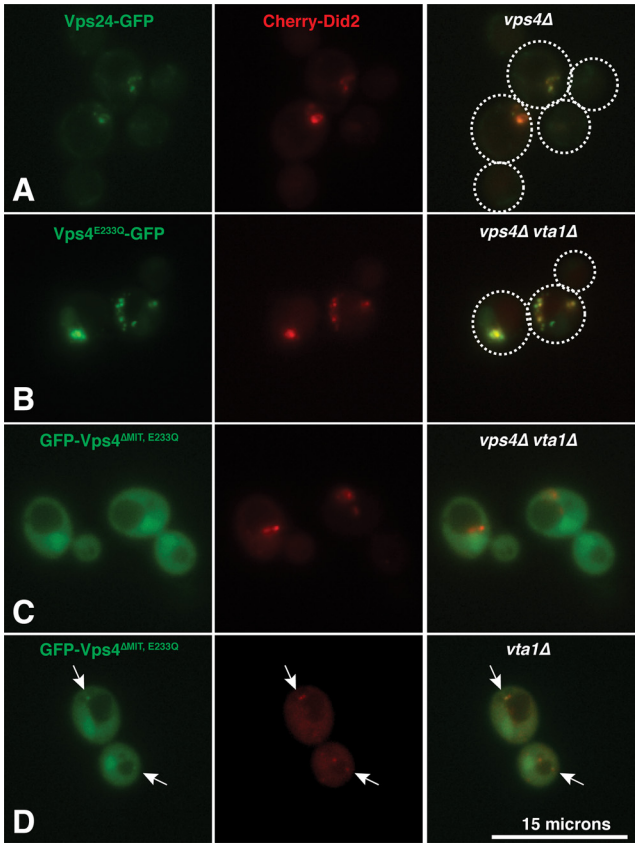

Figure 5. Vps4$^{\Delta MIT,E233Q}$ colocalization with Did2 is Vps4-dependent. (A) In yeast lacking Vps4 (*vps4*Δ), Cherry-Did2 exhibits colocalization with the core ESCRT-III subunit Vps24-GFP. Dashed white lines represent the borders of individual yeast cells. Bar, 15 μm. (B) Vps4^{E233Q}-GFP exhibits punctate distribution and colocalizes extensively with Cherry-Did2 in *vps4*Δ *vta1*Δ cells. (C) GFP-Vps4$^{\Delta MIT,E233Q}$ exhibits a diffuse distribution and little colocalization with Cherry-Did2 in *vps4*Δ *vta1*Δ cells. (D) In the presence of Vps4 (*vta1*Δ cells), GFP-Vps4$^{\Delta MIT,E233Q}$ displays some punctate association that colocalizes with Cherry-Did2 (indicated by arrows). Additional micrographs demonstrating colocalization of GFP-Vps4$^{\Delta MIT,E233Q}$ with Cherry-Did2 in the presence of wild-type Vps4 are presented in Supplemental Figure 4.

in which Vps4 oligomer dissociation is implicit within the ATP hydrolysis cycle.

To address the functional stability of the Vps4 oligomer in the presence of ESCRT-III, the ability of Vps4^{E233Q} to inhibit Vps4 disassembly activity (Figure 6A) was exploited. If Vps4 undergoes dynamic oligomerization and dissociation during ESCRT-III disassembly, then addition of Vps4^{E233Q} subunits subsequent to initiation of the reaction should arrest the ESCRT-III disassembly process; however, if Vps4 functions as a stable oligomer, then Vps4 disassembly activity may be resistant to subsequent addition of Vps4^{E233Q}. Reactions were initiated with Vps4 and ATP, then Vps4^{E233Q} was added 5 min after initiation, and the effect on ESCRT-III disassembly was assessed. Whereas premixing of Vps4 and Vps4^{E233Q} inhibited ESCRT-III disassembly, addition of Vps4^{E233Q} subsequent to initiation failed to arrest Vps4 activity (Figure 7C). This result indicated that Vps4^{E233Q} inhibition of ESCRT-III disassembly was sensitive to the time of addition. This result also suggested that the active Vps4 oligomer was resistant to Vps4^{E233Q} inhibition and that exchange of Vps4 subunits with the soluble pool did not occur once the active oligomer formed. While we cannot exclude that additional factors modulate the dynamics of Vps4 oligomerization in vivo, these results are consistent with the conclusion that the Vps4 oligomer is stable during ATP hydrolysis both in the absence and presence of ESCRT-III and suggest that the Vps4 oligomer remains intact during ESCRT-III disassembly.

DISCUSSION

ESCRT-III disassembly by Vps4 ATP hydrolysis plays a key role in MVB sorting, although how this process occurs and how this reaction contributes to ILV formation remain poorly defined. In vitro reconstitutions of ESCRT-III subunits binding to membranes have demonstrated the principles that the ESCRT-III subunits themselves can deform the membrane and accomplish membrane scission even in the absence of Vps4 or the other ESCRTs (Saksena *et al.*, 2009; Wollert *et al.*, 2009). Whether Vps4 functions subsequent to ESCRT-III–mediated ILV budding to recycle ESCRT-III subunits (Wollert *et al.*, 2009) or whether Vps4 also plays an active role rearranging ESCRT-III during ILV budding are issues that remain to be resolved. Two general models for Vps4 remodeling of ESCRT-III have emerged: 1) Vps4 undergoes a dynamic cycle of oligomerization upon ATP binding and hydrolysis, with ESCRT-III disassembly linked to Vps4 dissociation; or 2) Vps4 functions as a stable oligomer with ATP hydrolysis facilitating ESCRT-III function independent of Vps4 dissociation. The functional studies presented here support models in which Vps4 functions as a stable oligomer during ESCRT-III disassembly. In addition, these studies demonstrated that neither a full complement of MIT domains nor concerted ATP hydrolysis by all subunits are required for the Vps4 oligomer to support ESCRT-III disassembly. These studies suggest a model wherein ESCRT-III engagement (via the MIT domain) and ATP hydrolysis are coordinated at the subunit level within the Vps4 oligomer, leading to disassembly of the ESCRT-III polymer by the stable Vps4 oligomer in a processive manner.

Vps4 is a type 1 AAA-ATPase that forms an active oligomer in the presence of ATP (Babst *et al.*, 1998). Reconstructions of electron micrographs have suggested that the Vps4^{E233Q} oligomer consists of two conformationally distinct hexameric rings (Yu *et al.*, 2008; Landsberg *et al.*, 2009), analogous to type 2 AAA-ATPases including p97 and NSF (White and Lauring, 2007). Size exclusion chromatography of Vps4 and Vps4^{E233Q} has implicated ATP hydrolysis in rapidly dissociating the Vps4 oligomer (Babst *et al.*, 1998). This feature along with the potential of the Vps4 oligomer to concurrently engage multiple ESCRT-III subunits via MIT domains have suggested a possible mechanism for Vps4-mediated ESCRT-III disassembly (Babst *et al.*, 1998; Landsberg *et al.*, 2009). However, another model has emerged through investigations of the central pore of the Vps4 oligomeric ring (Scott *et al.*, 2005a; Gonciarz *et al.*, 2008). Mutational analyses of residues lining this central cavity have suggested that the Vps4 pore facilitates substrate remodeling in a manner analogous to other AAA-ATPases such as ClpX, Hsp104 and NSF (Hanson and Whiteheart, 2005; White and Lauring, 2007). This model implies that Vps4 functions as a stable oligomer facilitating ESCRT-III disassembly via the pore, although neither the ability of Vps4 to alter ESCRT-III subunit conformations nor the stability of the Vps4 oligomer during ESCRT-III disassembly had been demonstrated. To explore these two distinct models of Vps4 function and to gain insights into coordination among subunits within the Vps4 oligomer, the Vps4-mediated ESCRT-

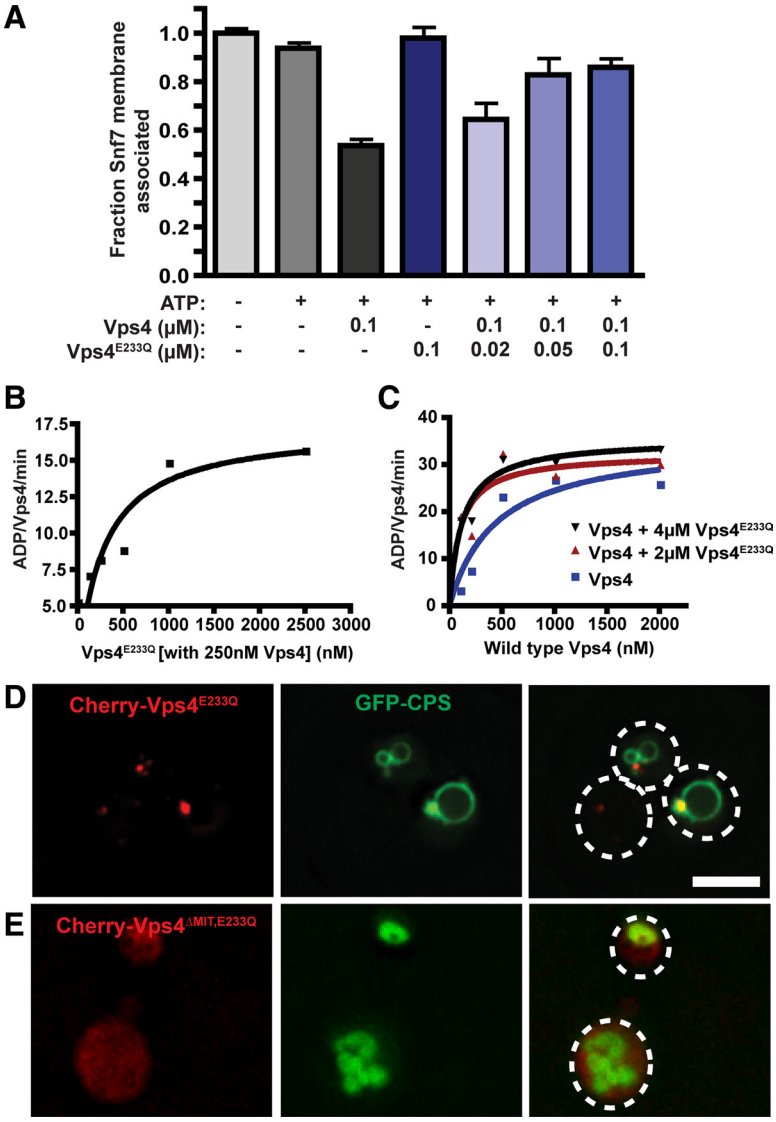

Figure 6. Vps4^{E233Q} inhibition of ESCRT-III disassembly. (A) ESCRT-III disassembly reactions were performed with *vps4*Δ membranes (0.5 OD_{600} equivalents) and 100 nM Vps4, 100 nM Vps4^{E233Q}, or 100 nM Vps4 with decreasing concentrations of Vps4^{E233Q} (100 nM, 50 nM, and 20 nM). (B) ATP hydrolysis reaction were performed with 250 nM Vps4 and increasing concentrations of Vps4^{E233Q}. The initial ATP concentration was 1 mM. Rates are presented as ADP generated per wild-type Vps4 per minute. (C) ATP hydrolysis reactions were initiated with 2 μM Vps4^{E233Q} (red), 4 μM Vps4^{E233Q} (black), or Vps4 alone (blue) with Vps4 concentrations from 100 nM to 2 μM. Rates are presented as ADP generated per wild-type Vps4 per minute. (D and E) Distribution of the MVB sorting reporter GFP-CPS in wild-type yeast expressing Cherry-Vps4^{E233Q} (D) or Cherry-Vps4$^{\Delta MIT,E233Q}$ (E). Dashed white lines represent the borders of individual yeast cells. Bar, 5 μm.

III disassembly reaction was reconstituted in vitro using ESCRT-III polymers generated in yeast. This system also permitted examination of the ESCRT-III subunit-dependence of this disassembly process.

ESCRT-III subunits have been observed to assemble into fibril-like polymers in vivo that may spiral to facilitate membrane deformation and ILV budding (Hanson *et al.*, 2008). Snf7 appears to be the predominant component of these ESCRT-III fibrils (Hanson *et al.*, 2008; Teis *et al.*, 2008) and was selected as the primary indicator of ESCRT-III membrane association and assembly in this reconstituted system. While the individual losses of Vps20, Ist1, or Vps60 did not perturb Snf7 membrane release, the absences of Vps24, Vps2, or Did2 compromised the ESCRT-III disassembly reaction with the loss of Vps2 associated with the most severe effect. These observations recapitulate prior studies indicating that Snf7 recycling in vivo is dependent upon Vps24, Vps2, and Did2 (Babst *et al.*, 2002a; Nickerson *et al.*, 2006; Teis *et al.*, 2008), supporting the suitability of this system for investigating Vps4 disassembly of ESCRT-III. While Vps24, Vps2, and Did2 may directly contribute to the recruitment, activation, and/or progression of Vps4 during ESCRT-III disassembly, it is also possible that the observed effects are indirect. For instance, the absences of these subunits (Vps24, Vps2 or Did2) may permit Snf7 subunits to assemble in an aberrant manner recalcitrant to Vps4 disassembly. Another possibility is that losses of these subunits may permit other factors to bind to ESCRT-III that impinge on Vps4 activity. While further investigation of this system is required to address these possibilities and to define the individual contributions, the experiments presented herein demonstrate that Vps24, Vps2 and Did2 facilitate Vps4 disassembly of ESCRT-III.

These studies also imply that the Vps20-Snf7 oligomer is resistant to Vps4 dissociation in spite of the presence of a Vps4 interaction motif (MIM2). This suggests that the MIM1-mediated activation of Vps4 ATPase activity is critical for ESCRT-III dissociation. Thus, maturation of the ESCRT-III polymer with the incorporation of MIM1-containing subunits may serve both to permit completion of ILV formation (Wollert *et al.*, 2009) and to facilitate Vps4-mediated ESCRT-III disassembly, thereby providing a timing component for ESCRT-III function (Supplemental Figure 4C). The role of the Vps2 MIM1 element in ESCRT-III function was investigated, as loss of Vps2 could not be suppressed by increasing the Vps4 concentration. A Snf7-Vps2 chimera (Snf7^{Vps2MIM1})

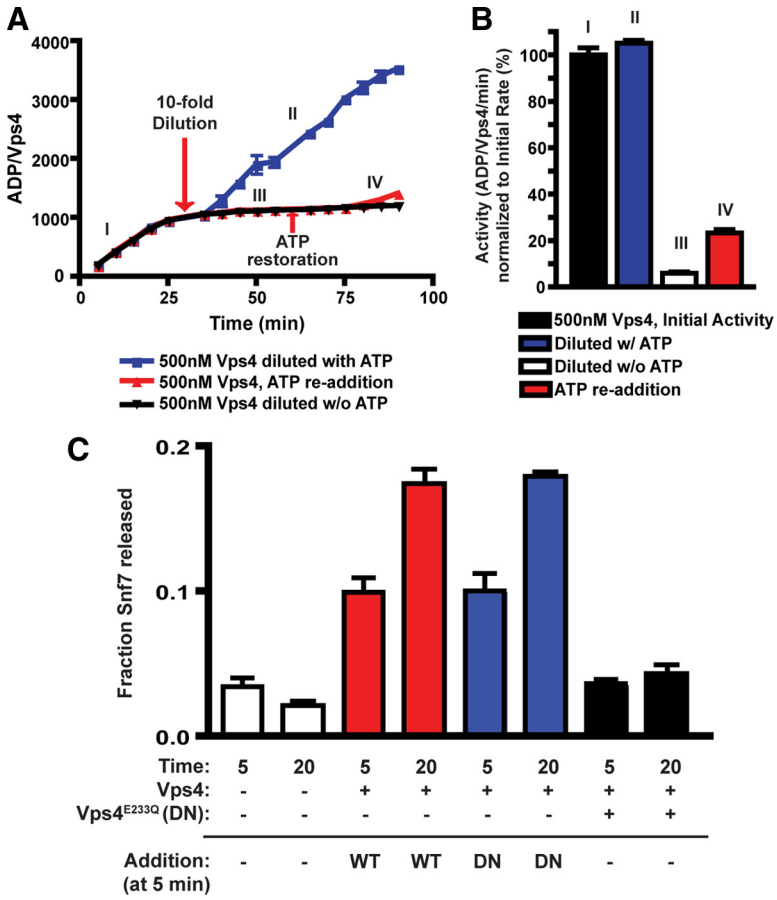

Figure 7. The Vps4 oligomer is functionally stable in vitro. (A) ATP hydrolysis reactions were initiated with 500 nM Vps4 and 1 mM ATP. After 30 min, reactions were diluted 10-fold in the absence of additional ATP (black, red) or with ATP maintained at 1 mM (blue). After an additional 30 min, ATP restoration (to 1 mM, red) was performed. The ADP generated per Vps4 was determined at 5-min intervals and plotted versus time. (B) The activities of ATP hydrolysis (ADP generated/Vps4/min) relative to the initial rate (I) are presented. (C) ESCRT-III release reactions were initiated with 30 nM Vps4 (red, blue) or with 30 nM Vps4 and 100 nM $Vps4^{E233Q}$ (black). After a 5-min incubation, Vps4 (30 nM, red) or $Vps4^{E233Q}$ (100 nM, blue) were added to the reactions initiated with Vps4 only. The fraction of Snf7 released into the soluble material was assessed at both 5 and 20 min.

was examined for function in the ESCRT-III disassembly system as well as for effects on MVB sorting in vivo. $Snf7^{Vps2MIM1}$ was competent as a substrate for disassembly by Vps4 and could bypass the requirement for Vps2 in this system; this observation is consistent with the ability of the Vps2 MIM1 to enable Vps4 disassembly of $Vps24^{Vps2MIM1}$ homo-oligomers (Ghazi-Tabatabai *et al.*, 2008). However, $Snf7^{Vps2MIM1}$ was not able to complement the MVB sorting defects of *snf7*Δ yeast and perturbed MVB sorting of wild-type cells. These results indicate that it is possible to separate the requirements for ESCRT-III function in MVB sorting from the requirements for Vps4-mediated ESCRT-III disassembly, although this distinction is accomplished via a non-native protein. $Snf7^{Vps2MIM1}$ may enable the premature disassembly of ESCRT-III, disrupting the coordination of ESCRT-III assembly and disassembly necessary for MVB sorting. However, alternative possibilities, including indirect effects on ESCRT-III assembly or the altered recruitment of ESCRT-III effectors, may also contribute to the disruptive effects of $Snf7^{Vps2MIM1}$. Further investigation will be required to understand the coordination of ESCRT-III assembly and disassembly that facilitates MVB sorting.

The ESCRT-III disassembly assay was also used to examine the functional requirements for Vps4. Both ATP hydrolysis and ESCRT-III engagement via the MIT domain were required for Vps4 to mediate ESCRT-III disassembly. The in vitro system permitted these requirements to be examined further through mixing wild-type and mutant subunits, addressing functional aspects of the Vps4 oligomer. Addition of equimolar or excess $Vps4^{\Delta MIT}$ or $Vps4^{\Delta MIT,E233Q}$ subunits did not compromise the ESCRT-III disassembly function of wild-type Vps4 subunits. Moreover, an excess of $Vps4^{\Delta MIT,E233Q}$ was able to enhance the activity of a limiting amount of Vps4. These observations indicate that subunits lacking the MIT domain can be incorporated into a Vps4 oligomer without abolishing oligomer activity and that the Vps4 oligomer does not require a full complement of MIT domains. The observations with $Vps4^{\Delta MIT,E233Q}$ subunits also indicate that concerted ATP hydrolysis by all subunits of the Vps4 oligomer is not required for ESCRT-III disassembly. The demonstration that even a single catalytically active subunit can enable function of the hexameric AAA-ATPase ClpX (Hersch *et al.*, 2005; Martin *et al.*, 2005) is consistent with this conclusion that concerted ATP hydrolysis by all subunits is not required for Vps4 function; however, additional refinement of the hydrolysis requirements for the Vps4 oligomer will be required to permit further comparisons with the enzymology and mechanisms of ClpX (Glynn *et al.*, 2009). Consistent with these in vitro observations of Vps4, $Vps4^{\Delta MIT,E233Q}$ was observed to localize to endosomes in the presence of wild-type Vps4 subunits without disrupting MVB sorting, consistent with recently reported studies (Shestakova *et al.*, 2010). While the incorporation of $Vps4^{\Delta MIT}$ or $Vps4^{\Delta MIT,E233Q}$ subunits are anticipated to alter the Vps4 oligomer affinity for ESCRT-III and/or the efficiency of ESCRT-III disassembly, these effects were not apparent under the conditions examined. Further examination of these properties is warranted, but the results presented here establish the principles that neither a full complement of MIT domains nor concerted ATP hydrolysis by all subunits are required for ESCRT-III disassembly by the Vps4 oligomer.

While concerted hydrolysis by all subunits is not required, the coordination of ESCRT-III binding via the MIT domain with ATP hydrolysis by the engaged subunit appears to be a critical aspect of Vps4 function. Hydrolysis-defective mutants of Vps4, such as Vps4^{E233Q}, have previously been exploited as dominant negative mutants disrupting MVB sorting, viral budding and abscission (Babst *et al.*, 1997; Garrus *et al.*, 2001; Carlton and Martin-Serrano, 2007). However, the basis of compromised Vps4 oligomer function was not previously addressed. In contrast to the dominant negative effect in vivo, Vps4^{E233Q} subunits enhanced the ATPase activity of wild-type subunits in vitro. A similar stimulation by noncatalytic subunits has been observed with Hsp104/ClpB AAA-ATPases by treating with a nonhydrolyzable ATP analog, ATPγS (Doyle *et al.*, 2007); however, ATPγS treatment inhibited Vps4 activity in a manner comparable to the behavior of the AAA-ATPase ClpA rather than the stimulation observed with Hsp104 and ClpB (data not shown and Doyle *et al.*, 2007). Whereas Vps4^{E233Q} subunits enhanced ATPase activity of wild-type subunits, Vps4^{E233Q} addition disrupted Vps4 ESCRT-III disassembly even with Vps4 in excess. The distinct effects of Vps4^{E233Q} and Vps4$^{\Delta MIT,E233Q}$ on ESCRT-III disassembly indicated that the inhibitory effect of Vps4^{E233Q} is dependent on the MIT domain, and this dependence is similarly observed for Vps4^{E233Q} inhibition of MVB sorting (Figure 6D and Shestakova *et al.*, 2010). These results suggest that coordination between MIT engagement and ATP hydrolysis is required at the subunit level within the Vps4 oligomer. One possibility is that ESCRT-III binding via the MIT domain induces a conformational change within the engaged Vps4 subunit that must be resolved by ATP hydrolysis for the Vps4 oligomer to continue to disassemble ESCRT-III. Another nonexclusive possibility is that ESCRT-III binding via the MIT domain facilitates transfer of ESCRT-III to the central pore and that ATP hydrolysis by the MIT-engaged Vps4 subunit must occur to prevent occlusion of the pore. Moreover, these two possibilities may be related and serve to orchestrate substrate engagements via the MIT domain and the central pore with ATP hydrolysis. While the analysis presented here indicates that coordination of MIT domain engagement and ATP hydrolysis occurs at the subunit level within the Vps4 oligomer, the mechanisms by which this occurs remains to be determined.

The ability of Vps4^{E233Q} to inhibit ESCRT-III disassembly enabled the dynamics of Vps4 oligomerization to be examined. Whereas premixing of Vps4^{E233Q} with Vps4 inhibited ESCRT-III disassembly, addition of Vps4^{E233Q} subsequent to initiation of the reaction prevented the inhibitory effect. This result suggests that dynamic exchange of Vps4 subunits does not occur during the in vitro ESCRT-III disassembly reaction and that Vps4 functions as a stable oligomer. Moreover, these results suggest that a Vps4 oligomer may remain associated with an ESCRT-III polymer as it disassembles ESCRT-III subunits in a processive manner (rather than the Vps4 oligomer releasing and rebinding to the ESCRT-III

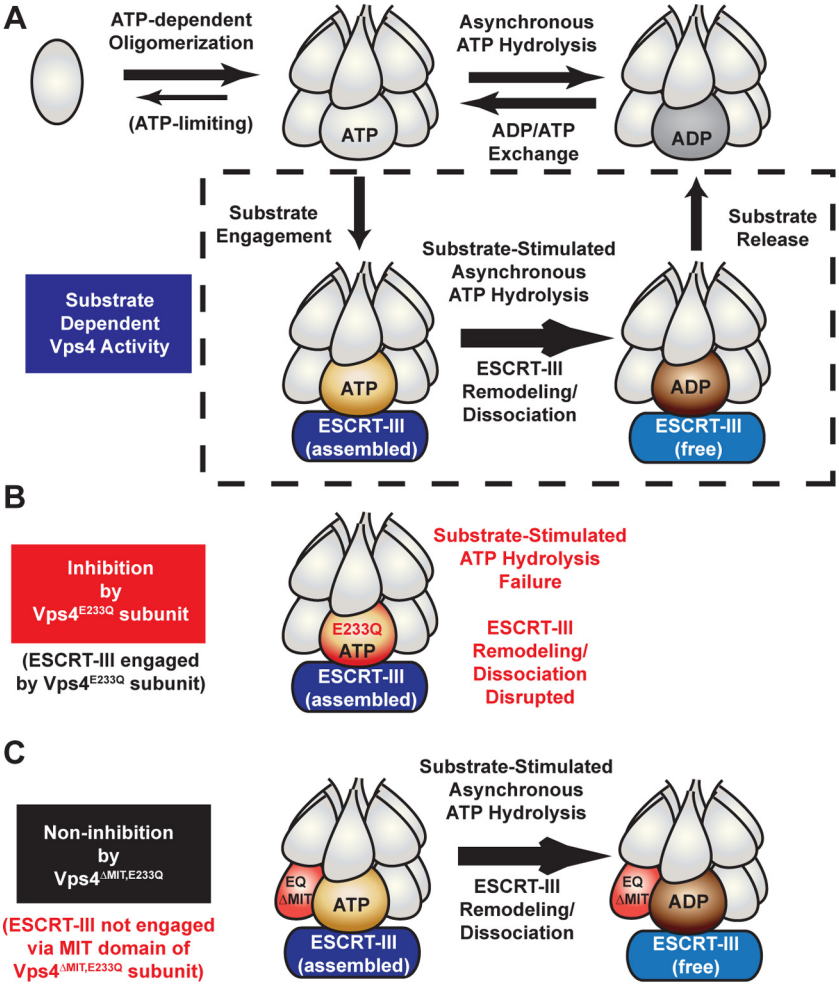

Figure 8. Coordination of Vps4 ESCRT-III engagement and ATP hydrolysis at the subunit level. (A) Vps4 ATP hydrolysis cycle in the presence or absence of the substrate ESCRT-III as suggested by in vitro analyses. Vps4 oligomerizes in an ATP-dependent manner, suggested to form a two-ring dodecamer (Yu *et al.*, 2008; Landsberg *et al.*, 2009). Neither concerted hydrolysis nor dissociation appear requisite for ATPase activity (upper row) or ESCRT-III disassembly (lower row), suggesting that individual subunits within the oligomer may hydrolyze and reload ATP. The presence of the substrate ESCRT-III can enhance Vps4 ATPase activity in an MIT-dependent manner, suggesting that engagement via the MIT domain potentiates ATP hydrolysis at the Vps4 subunit level to remodel and release ESCRT-III subunits (lower row). [Please note that the orientation of the asymmetric Vps4 oligomer relative to substrate is depicted with artistic license rather than adherence to supportive biochemical observations.] (B) If the engaged Vps4 subunit cannot hydrolyze ATP (Vps4^{E233Q}), then ESCRT-III disassembly is arrested. However, Vps4^{E233Q} does not disrupt substrate-independent ATPase activity (upper row in A). (C) The inhibition of ESCRT-III disassembly by Vps4^{E233Q} is dependent upon the MIT domain, suggesting that coordination between ESCRT-III engagement via the MIT domain and ATP hydrolysis occurs at the subunit level within the Vps4 oligomer.

polymer). The stability of the Vps4 oligomer during ESCRT-III disassembly is consistent with the dilution-resistance of ATPase activity in vitro. While we cannot exclude the possibility that the dynamics of Vps4 oligomerization are altered by additional factors in vivo and we acknowledge the absence of direct biophysical demonstration of Vps4 oligomer stability in vitro, these results support a model wherein Vps4 assembles into an oligomer and facilitates ESCRT-III disassembly without itself dissociating. This model is consistent with asynchronous/nonconcerted ATP hydrolysis among Vps4 subunits, whereby the remaining ATP-bound subunits stabilize the oligomer to permit ATP reloading after hydrolysis without dissociation (Figure 8A). This model is supported by the observation that catalytically inactive subunits ($Vps4^{E233Q}$) stimulate the ATPase activity of wild-type subunits in vitro in the absence of substrate. ESCRT-III binding has been demonstrated to enhance Vps4 ATPase activity in a MIT-dependent manner (Azmi *et al*., 2008). This observation in conjunction with the MIT-dependence of $Vps4^{E233Q}$ inhibition suggests that ESCRT-III binding via the MIT domain induces an activated state of the Vps4 oligomer with stimulation occurring at the level of the engaged subunit (Figure 8A). ATP hydrolysis by the engaged subunit is then required to facilitate ESCRT-III disassembly and permit continued Vps4 function (Figure 8B). The observation that the Vps4 oligomer does not require a full complement of MIT domains suggests that this cycle of ESCRT-III polymer binding, Vps4 subunit activation, ATP hydrolysis, and ESCRT-III subunit release occurs in a progressive manner to disassemble the ESCRT-III polymer rather than in mass. This cycle appears consistent with Vps4 remodeling ESCRT-III subunits via the central pore in a subunit-by-subunit manner to facilitate ESCRT-III disassembly, although experimental evidence will be required to confirm this model.

ACKNOWLEDGMENTS

We thank Dr. Zhaohui Xu (U. Michigan) for discussions regarding the Vps4 oligomer, Drs. Wesley Sundquist and Jack J. Skalicky (U. Utah) for discussions concerning Vps4 MIT-MIM2 interactions, members of the Katzmann and Horazdovsky labs for helpful scientific discussions, and Sandra Severson for technical support. This work has been supported by a Fraternal Order of Eagles postdoctoral fellowship (to B.A.D.), an American Heart Association predoctoral fellowship (AHA07-155882 to I.F.A.), and by grants R01 GM073024 (to D.J.K.) and R01 GM074171 (to M. B.) from the National Institutes of Health.

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