

Regulation of membrane scission in yeast endocytosis

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Abstract

Introduction

Clathrin-mediated endocytosis (CME) is the major endocytic process by which cargo from the cell exterior is incorporated into a Clathrin-coated vesicle that is then transported into the cell interior (*Bitsikas et al., 2014*). Over 50 different proteins are involved in reshaping a flat plasma membrane into an invagination that eventually forms the vesicle (*Kaksonen and Roux, 2018*). Forces that drive the transition from invagination to spherical vesicle in multicellular eukaryotes are provided by the GTPase Dynamin (*Grigliatti et al., 1973; Sweitzer and Hinshaw, 1998; Ferguson et al., 2007; Takei et al., 1995; Galli et al., 2017*). Dynamin is now known to interact via its proline-rich-domain with SH3 domains of crescent-shaped N-BAR proteins like Endophilin and Amphiphysin (*Grabs et al., 1997; Cestra et al., 1999; Farsad et al., 2001; Ferguson et al., 2009; Meinecke et al., 2013*). Conformation changes of Dynamin recruited to N-BAR molecules cause constriction of the underlying invaginated membrane, resulting in vesicle formation (*Shupliakov et al., 1997; Zhang and Hinshaw, 2001; Zhao et al., 2016*).

In yeast, CME is the only pathway for uptake of cargo, and involves a similar membrane transformation as in other eukaryotes. Most mammalian CME proteins have homologues in yeast: these proteins drive the establishment of endocytic sites, form the mechanical link between membrane and actin proteins (*Kaksonen and Roux, 2018*). Actin nucleation and polymerization drives the formation of tubular invaginations in yeast (*Kübler et al., 1993; Kaksonen et al., 2003*). The role of Dynamin in this process has been debated: yeast dynamin-like protein Vps1 has a major role in the Golgi and other membrane trafficking pathways (*Rothman et al., 1990; Peters et al., 2004; Hoepfner et al., 2001*), and been proposed to interact with endocytic proteins (*Nannapaneni et al., 2010; Yu and Cai, 2004; Smaczynska-de Rooij et al., 2012*). Its contribution to CME is however, still debated (*Goud Gadila et al., 2017; Kishimoto et al., 2011*). In yeast cells, what causes membrane scission is thus unclear, although the yeast N-BAR Rvs complex (a heterodimeric complex of the proteins Rvs161 and Rvs167) has been identified as an important component of the scission module (*Munn et al., 1995; Kaksonen et al., 2005; D'Hondt et al., 2000; Kishimoto et al., 2011*). The two Rvs proteins are homologues of N-BAR proteins Amphiphysin and Endophilin (*Friesen et al., 2006; Youn et al., 2010*). Deletion of Rvs167 reduces scission efficiency by nearly 30% and reduces the invagination lengths at which scission occurs (*Kaksonen et al., 2005; Kukulski et al., 2012*). Apart from the canonical N-BAR domain which forms the crescent-shaped structure, Rvs167 has a Glycine-Proline-Alanine rich (GPA) region and a C-terminal SH3 domain (*Sivadon et al., 1997*). The GPA region is thought to act as a linker with no other known function, while loss of the SH3 domain affects budding pattern and actin morphology (*Sivadon et al., 1997*). Most Rvs deletion phenotypes

43 can be rescued by expression of the BAR domains alone (*Sivadon et al., 1997*), suggesting that the
44 BAR domains are the functional unit of the Rvs complex.

45 The Rvs complex can tubulate liposomes in vitro, indicating that the BAR domains can impose
46 curvature on membranes (*Youn et al., 2010*). However, Rvs arrives at endocytic sites when mem-
47 brane tubes are already formed: curvature sensing rather than generation is the likely interaction
48 of the complex with endocytic sites (*Kukulski et al., 2012; Picco et al., 2015*). Rvs molecules arrive
49 at endocytic sites about 4 seconds before scission, and disassemble rapidly at the time of scission
50 (*Picco et al., 2015*), consistent with a role in scission. While it is shown to be involved in the last
51 stages of endocytosis, a mechanistic understanding of the influence of Rvs on scission remains
52 incomplete.

53
54 Several scission models have been proposed that allow a major role for Rvs and are tested in
55 this work. Although the yeast Dynamin Vps1 lacks a canonical BAR-protein binding site (*Bui et al.,*
56 *2012; Moustaq et al., 2016*), it may be recruited via a different mechanism and induce scission.
57 Liu et al., proposed that Synaptojanins may selectively hydrolyze lipids at endocytic sites, causing
58 line tension between two lipid types that results in scission (*Liu et al., 2009*). Protein friction
59 along the membrane invagination has been proposed as a mechanism by which scission may
60 occur (*Simunovic et al., 2017*). We used quantitative live-cell imaging and genetic manipulation in
61 *Saccharomyces cerevisiae* to test these theories and investigate the function of Rvs in endocytosis.
62 We found that Rvs is recruited to endocytic sites by both BAR and SH3 domains. Of several potential
63 actin-interacting binding partners of the SH3 domains such as Myo3, Myo5, Vrp1, Abp1 (*Lila and*
64 *Drubin, 1997; Colwill et al., 1999; Madania et al., 1999; Liu et al., 2009*), we found that type I myosin
65 Myo3 interacts with Rvs SH3 domains. Our data also suggests that the aforementioned theories
66 of membrane scission are unlikely to sever the membrane in yeast, and that actin polymerization
67 likely generates the forces required for scission.

68 Results

69 **Rvs167, rather than Vps1 influences coat movement**

70 Yeast Dynamin-like protein Vps1 does not contain a Proline Rich Domain, which in mammalian
71 cells is required for recruitment to endocytic sites (*Grabs et al., 1997; Cestra et al., 1999; Farsad*
72 *et al., 2001; Meinecke et al., 2013*). In spite of the lack of a stereotypical interaction domain, some
73 works have reported its recruitment to endocytic proteins, including to N-BAR protein Rvs167 (*Yu*
74 *and Cai, 2004; Nannapaneni et al., 2010; Rooij et al., 2010*). The question of whether or not Vps1
75 has a function at endocytic sites has been obfuscated by potential tagging-induced dysfunction
76 of Vps1 molecules. Vps1 tagged both N- and C-terminally with GFP constructs failed to co-localize
77 with endocytic protein Abp1 in our hands, consistent with other work that observed localization
78 only with other parts of the trafficking pathway (*Goud Gadila et al., 2017*). We argued that even
79 if tagging Vps1 induced defects in its localization and/or function, its contribution to endocytosis
80 could be examined by observing the dynamics of other endocytic proteins in cells lacking Vps1. The
81 canonical interaction partner of Vps1- Rvs167- localizes to endocytic sites, and has a role in scission,
82 although it is unclear what that is (*Kukulski et al., 2012; Picco et al., 2015*). In order to determine
83 the roles of these proteins in endocytic scission, we studied cells lacking Vps1 and Rvs167, and
84 compared against wild-type (WT) cells (Fig.1A-F).

85
86 Vps1 deletion was confirmed by sequencing the gene locus, and these cells showed a previously
87 reported (*Rothman and Stevens, 1986*) growth phenotype at high temperatures (Fig.S1). Scission
88 efficiency was quantified by tracking the endocytic coat protein Sla1 tagged at the C-terminus with
89 eGFP (Fig.1C). Upon actin polymerization, the endocytic coat moves into the cytoplasm along with
90 the membrane as it invaginates (*Skruzny et al., 2012*). Movement of coat protein Sla1 thus acts as a
91 proxy for the growth of the plasma membrane invagination. Membrane retraction, that is, inward

92 movement and subsequent retraction of the invaginated membrane back towards the cell wall is a
 93 scission-specific phenotype (*Kaksonen et al., 2005*). Retraction rates do not significantly increase in
 94 *vps1Δ* cells compared to the WT (Fig.1C).

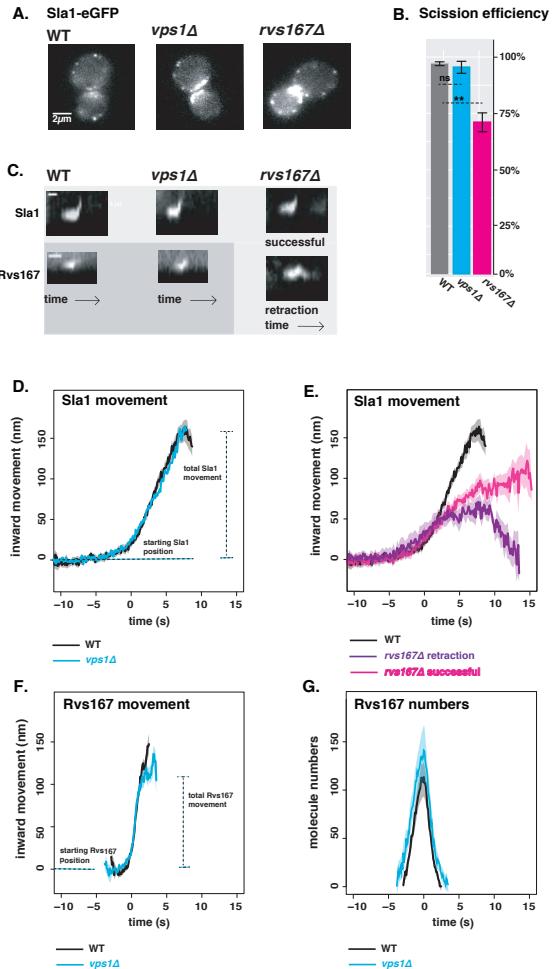


Figure 1. *vps1Δ* and *rvs167Δ* deletion **A:** Single slices from time lapse movies of WT, *vps1Δ*, and *rvs167Δ* cells expressing Sla1-eGFP. Scale bar= 2μm. **B:** Representative kymographs of Sla1-eGFP and Rvs167-eGFP patches in WT, *vps1Δ*, and *rvs167Δ* cells from time lapse movies. Scale bar for Sla1-egfp = 20(s), scale bar for Rvs167-eGFP = 5(s). **C:** Scission efficiency in WT, *vps1Δ*, and *rvs167Δ* cells. Error bars are standard deviation, p values from t-test, * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001. **D:** Averaged centroid positions of Sla1-eGFP in WT and *vps1Δ* cells. **E:** Averaged position of Rvs167-eGFP in WT and *vps1Δ* cells. **F:** Averaged position of Sla1-eGFP in WT, and successful and retracted Sla1-eGFP positions in *rvs167Δ* cells. All averaged positions were aligned in x axis to begin inward movement at time=0(s), and aligned in the y axis to a starting position = 0(nm).

95
 96 The total movement of the endocytic coat (Fig.1D,E) gives an indication of when invagination has
 97 undergone scission: greater movement would imply defects in the scission mechanism. In order
 98 to study this movement , the averaged centroid trajectory of 50 Sla1-eGFP patches in *vps1Δ* and
 99 WT cells were tracked and compared (Fig.1D). In brief: yeast cells expressing fluorescently-tagged
 100 endocytic proteins were imaged at the equatorial plane. Since membrane invagination progresses
 101 perpendicularly to the plane of the plasma membrane, proteins that move into the cytoplasm
 102 during invagination do so in the imaging plane. Centroids of Sla1 patches- each patch being an
 103 endocytic site- were tracked in time and averaged. This provided an average centroid that could be

104 followed with high spatial and temporal precision (*Picco et al., 2015*). Averaged centroid movement
105 of Sla1-eGFP in WT cells was linear to about 140nm (Fig.1D). Sla1 movement in *vps1Δ* cells had the
106 same magnitude of movement (Fig.1D). In spite of slight differences in the rates of movement, the
107 total movement- and so the depth of endocytic invagination- did not change.
108
109 Centroid tracking has shown that the number of molecules of Rvs167 peaks at the time of scission,
110 and is followed by a rapid loss of fluorescent intensity, simultaneous with a sharp jump of the
111 centroid into the cytoplasm (*Picco et al., 2015*). This jump, also seen in Rvs167-GFP kymographs
112 (Fig.1B), is interpreted as loss of protein on the membrane tube, causing an apparent spatial jump
113 to the protein localized at the base of the newly formed vesicle. Kymographs of Rvs167-GFP (Fig.1B),
114 as well as Rvs167 centroid tracking (Fig.1E) in Vps1 deleted cells showed the same jump as in WT.
115
116 The Rvs complex is composed of Rvs161 and Rvs167 dimers (Boeke et al. 2014) so deletion of
117 Rvs167 effectively removes both proteins from endocytic sites. We quantified the effect of *rvs167Δ*
118 on membrane invagination (Fig.1A-C,F). Only 73% of Sla1 patches undergo successful scission in
119 *rvs167Δ* cells (Fig.1C). Similar scission rates have been measured in other experiments (*Kaksonen*
120 *et al., 2005*), and suggest failed scission in the remaining 27% of endocytic events. Coat movement
121 both of retractions and of successful endocytic events were quantified (Fig.1F) as described earlier.
122 Sla1 centroid movement in both successful and retracting endocytic events in *rvs167Δ* cells look
123 similar to WT up to about 50nm (Fig.1F). In WT cells, Abp1 intensity begins to drop at scission
124 time (Fig.S2); similarly, in successful endocytic events, Abp1 intensity drops after Sla1 centroid has
125 moved about 100nm suggesting that scission occurs at invagination lengths between 60 -100 nm
126 (Fig.S4). That membrane scission occurs at shorter invagination lengths than in WT is corroborated
127 by the smaller vesicles formed in *rvs167Δ* cells by Correlative light and electron microscopy (CLEM)
128 (*Kukulski et al., 2012*). CLEM has moreover shown that Rvs167 localizes to endocytic sites after
129 the invaginations are about 60nm long (*Kukulski et al., 2012*). Sla1 movement in *rvs167Δ* indicates
130 therefore that membrane invagination is unaffected till Rvs is supposed to arrive.

131 **Synaptojanins likely influence vesicle uncoating, but not scission dynamics.**

132 As Vps1 did not appear to influence membrane scission, we proceeded to test another scission
133 model. The lipid hydrolysis model proposes that deletion of yeast synaptojanins would inhibit
134 scission and therefore result in longer invaginations (*Liu et al., 2009*). Three Synaptojanin-like
135 proteins have been identified in *S. cerevisiae*: Inp51, Inp52, and Inp53. Inp51-eGFP exhibits a
136 diffuse cytoplasmic signal, Inp52-eGFP localizes to cortical patches that are endocytic sites (Fig.2A,
137 supplement) and Inp53 localizes to patches within the cytoplasm (Fig.2A, (*Bensen et al., 2000*)). Since
138 Inp52 localizes to endocytic sites, we began with determining the spatial and temporal recruitment
139 of Inp52 within the endocytic machinery. We aligned the averaged centroid of Inp52 in space and
140 time to other endocytic proteins (*Picco et al., 2015*). In order to do this, we imaged Inp52-eGFP
141 simultaneously with Abp1-mCherry, and did the same with Sla1-eGFP and Rvs167-eGFP. Using Abp1
142 as the common reference frame, we were able to compare the arrival of the different proteins with
143 respect to that of Abp1. We assigned as time =0 (s) the fluorescent intensity maximum of Abp1,
144 which in WT cells is concomitant with membrane scission, and also coincides with the maximum
145 of the Rvs167 fluorescent intensity (Fig.1A, S3). On the y axis, 0 (nm) indicates the position of the
146 Sla1 centroid; positions of the other centroids are in relation to the Sla1 centroid. Inp52 molecules
147 arrived in the late stage of endocytosis after Rvs167 arrival, and localized to the invagination tip,
148 suggesting a potential role in membrane scission (Fig.2B).

149 Inp53 was not investigated further, as its localization conformed with other literature that found
150 that it is involved in the golgi trafficking pathway and not endocytosis (*Bensen et al., 2000*). Although
151 we were unable to observe localization of Inp51 at endocytic sites, deletion of Inp51 has been
152 shown to exacerbate the effect of *inp52Δ* on membrane retraction (*Liu et al., 2009*), so both Inp51
153 and Inp52 were tested as potential scission regulators.

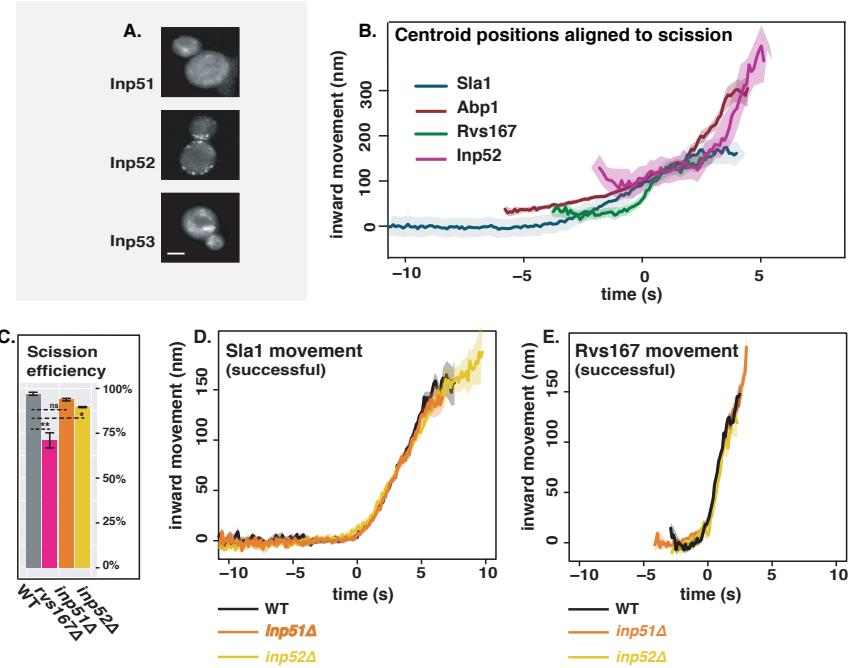


Figure 2. Involvement of yeast Synaptojanin-like proteins in endocytosis **A:** Cells endogenously tagged with Inp51-, Inp52-, and Inp53-eGFP. **B:** Inp52 centroid trajectory was aligned in space and time to other endocytic proteins. **C:** Sla1 retraction rates in *inp51Δ* and *inp52Δ* cells compared to WT and *rvs167Δ*. Error bars are standard deviation, with p values from t-test, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$. **D:** Averaged centroid positions of Sla1-eGFP in WT, *inp51Δ*, and *inp52Δ* cells. **E:** Averaged centroid positions of Rvs167-eGFP in WT, *inp51Δ*, and *inp52Δ* cells.

154

155

156 Dynamics of Sla1-eGFP and Rvs167-eGFP in *inp51Δ* and *inp52Δ* cells were compared against
 157 the WT (Fig.2C-E). Scission efficiency did not significantly decrease in *inp51Δ* compared to the WT,
 158 but showed a slight decrease in *inp52Δ* cells (Fig.2C). Total movement of Sla1 and Rvs167 centroids
 159 in *inp51Δ* were the same as in WT (Fig.2 D,E), while Rvs167 assembly and disassembly took longer
 160 (Fig.S5). Rvs167 centroid, after the inward movement, appeared to persist compared to the WT,
 161 likely because of a delay in Rvs167 disassembly from the newly formed vesicle. In *inp52Δ* cells,
 162 Sla1 movement had the same magnitude and rate as in WT, but Sla1-eGFP signal is persistent after
 163 inward movement scission (Fig.2D). Rvs167 and Sla1 disassembly were delayed in *inp52Δ* cells
 164 compared to WT (Fig.2supplement1). This data are consistent with Synaptojanin involvement in
 165 assembly and disassembly of coat and scission proteins at endocytic sites rather than in membrane
 166 scission.

167 **Rvs BAR domains recognize membrane curvature in-vivo**

168 So far Rvs167 remains the protein that has a major influence on scission efficiency and movement
 169 of Sla1. Rvs can tubulate liposomes in vitro (*You et al., 2010*), but its interaction with membrane
 170 curvature in vivo has not so far been tested. Recruitment of the Rvs complex to endocytic sites, and
 171 BAR-membrane interaction was thus investigated further. The SH3 domain has known interactions
 172 with proteins within actin network (*Lila and Drubin, 1997; Colwill et al., 1999; Madania et al., 1999;*
 173 *Liu et al., 2009*). We removed the contribution of the SH3 by deleting the domain (Fig.3A) and
 174 observed the localization of Rvs167 Δ sh3 compared to full-length Rvs167. Endogenously tagged
 175 Rvs167-eGFP and Rvs167 Δ sh3-eGFP colocalization with Abp1-mCherry in WT and *sla2Δ* cells were
 176 compared (Fig.3B). Sla2 acts as the molecular linker between forces exerted by the actin network

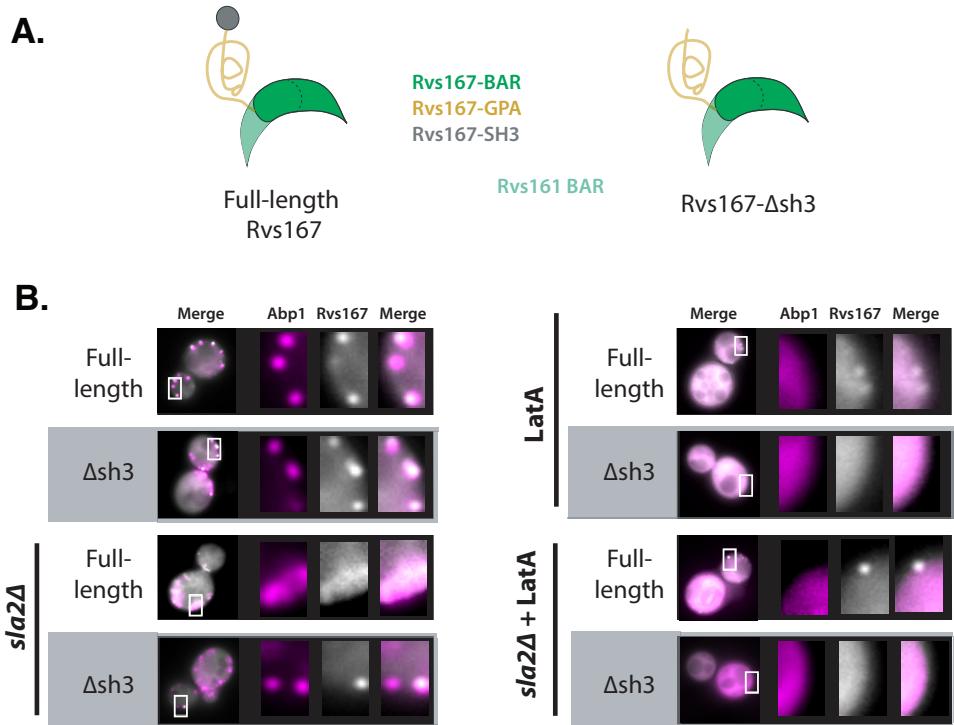


Figure 3. Localization of Rvs167 BAR domain **A:** Schematic of Rvs protein complex with and without the SH3 domain. **B:** Localization of full-length Rvs167 and Rvs167 Δ sh3 in WT, *sla2Δ*, LatA treated, and LatA treated *sla2Δ* cells. Scale bar=2 μ m.

177 and the plasma membrane (*Skrzyni et al., 2012*). *sla2Δ* cells therefore contain a polymerizing
 178 actin network at endocytic patches, but the membrane has no curvature, and endocytosis fails.
 179 In these cells, the full-length Rvs167 co-localizes with Abp1-mCherry, indicating that it is recruited
 180 to endocytic sites independently of membrane curvature (Fig.3B, “*sla2Δ*”). Rvs167 Δ sh3 does not
 181 localize to the plasma membrane except for rare transient patches that do not co-localize with
 182 Abp1-mCherry: Rvs167 Δ sh3 is not recruited to endocytic sites in the absence of curvature in *sla2Δ*
 183 cells.

184 **Rvs SH3 domains have an actin and curvature independent localisation**

185 In order to test if genetic interactions of SH3 domains are prevalent in *in vivo* endocytosis, we
 186 tested the localization of Rvs167 and Rvs167 Δ sh3 in LatA treated cells (Fig.3B, “*LatA*”). Plasma
 187 membrane localization of Rvs167 remains upon LatA treatment, and transient patches continue to
 188 exist in *sla2Δ* cells treated with LatA (Fig3B, “*sla2Δ + LatA*”). Rvs167 Δ sh3 does not localize to the
 189 plasma membrane in either case. Thus, localization of full-length Rvs167 in the presence of LatA is
 190 due to the SH3 domain. This indicates that the SH3 domain is able to recruit Rvs molecules to the
 191 plasma membrane in an actin- and curvature-independent manner.

192 **SH3 domains are likely recruited by Myosin 3**

193 Type I myosins Myo3 and Myo5, and Vrp1 have known genetic and/or physical interactions with
 194 Rvs167 SH3 domains (*Lila and Drubin, 1997; Colwill et al., 1999; Madania et al., 1999; Liu et al.,*
 195 **2009**). We tested the interaction between these proteins and the Rvs167 SH3 region by studying
 196 the localization of full-length Rvs167 in cells with one of the genes for these proteins deleted, and
 197 treated with LatA. By using LatA we expected to reproduce the situation in which BAR-curvature
 198 interaction is removed (Fig.4B). Then, if we lost SH3 interaction because we removed the protein

199 with which it interacts, we would lose localization of Rvs167 completely. Deletion of neither Vrp1
 200 nor Myo5 in combination with LatA treatment removes the localization of Rvs167. Deletion of
 201 Myo3 with LatA treatment removes localization of Rvs167, indicating that SH3 domains interact at
 202 endocytic sites with Myo3.

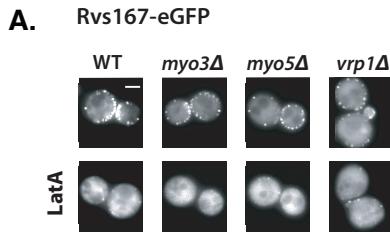


Figure 4. Localization of the SH3 domain Localization of full-length Rvs167-eGFP in WT, *myo3Δ*, *myo5Δ*, and *vrp1Δ* cells. Scale bars=2μm.

203 what about the differences in *myo5* and *myo3* number...

204 **Loss of Rvs167 SH3 domain affects coat and actin dynamics**

205 Since the Rvs167 SH3 domain has an influence on the recruitment of the Rvs complex to endocytic
 206 sites, we wondered if the domain also affects later stages of invagination formation endocytic
 207 dynamics. We compared dynamics of coat and scission markers in WT and *rvs167Δsh3* cells (Fig.5).
 208 Movement of Sla1 centroid is slower and reduced in *rvs167Δsh3* cells compared to WT (Fig4A,B).
 209 The movement of Rvs167textitΔsh3 centroid is smaller than that of full-length Rvs167 (Fig.5A,B),
 210 consistent with the formation of shorter invaginations suggested by the reduced Sla1 movement in
rvs167Δsh3 cells.

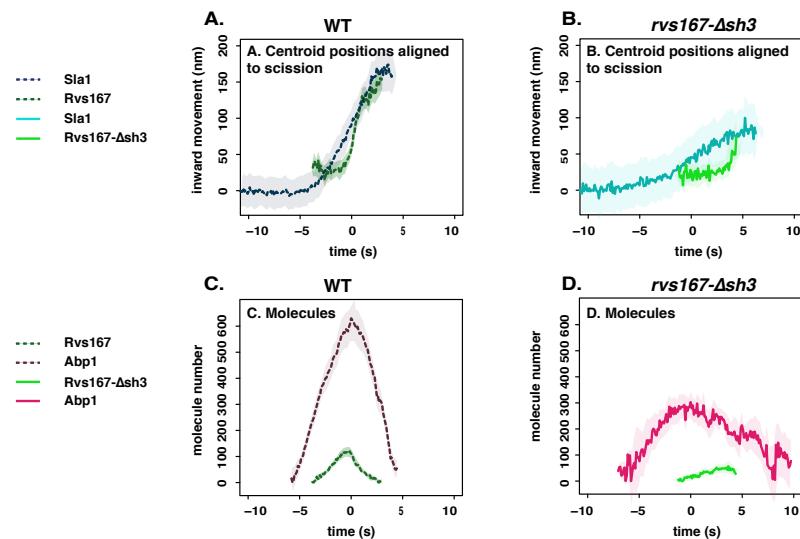


Figure 5. Endocytic dynamics in *rvs167Δsh3* cells **A,B:** Averaged centroid positions aligned in x axis so that time=0(s) is the peak of fluorescent intensity of Abp1 in respective strains. Centroids are aligned in y axis so that Sla1 begins at y=0 (nm), and Rvs167 and Rvs167Δsh3 positions are determined with respect to Sla1 centroids. **C,D:** Numbers of molecules of in WT and *rvs167Δsh3* cells, aligned so that time=0(s) is the maximum of fluorescent intensity of Abp1 in the corresponding strains.

211
 212 There is delay in Rvs167Δsh3 recruitment compared to the onset of Abp1 assembly in *rvs167Δsh3*
 213 cells compared to WT (Fig.5 C,D). In WT cells, Rvs167 and Abp1 molecule number peaks are also co-

214 incident: the actin network begins disassembling as soon as scission occurs (Fig.5C). Asynchronous
215 peaks in *rvs167Δsh3* cells indicates a disruption in the feedback between actin network dynamics
216 and membrane scission. Rvs167textitΔsh3 accumulation begins however, when Abp1 molecule
217 numbers in the mutant are about the same as in WT (about 300 copies, Fig.5C,D). . Both Rvs167
218 and Rvs167 Δ sh3 molecules arrive at endocytic sites when the Sla1 centroid is 20-30 nm away from
219 its starting position. This would mean the endocytic coat has moved about 30 nm when both WT
220 and mutant forms of Rvs are recruited. That Rvs167 Δ sh3 recruitment anticipates a certain growth
221 of the invagination and amount of Abp1 suggests that the Rvs complex is recruited to a specific
222 geometry of membrane invagination, and that Rvs167 Δ sh3 recruitment is delayed because invagi-
223 nations in these cells take longer to acquire this specific geometry. Recruitment of Rvs167 Δ sh3 is
224 reduced to half of Rvs167 (Fig.5C,D), although cytoplasmic concentration of both are similar (Fig.S6).
225 Recruitment therefore is unlikely to be limited by cytoplasmic expression of the mutant protein.
226 Abp1 disassembly is slowed down in *rvs167Δsh3* cells compared to WT, and recruitment is reduced
227 to 50% of WT recruitment (Fig.5C,D), indicating disruption of actin network dynamics.

228 **Increased BAR domain recruitment corresponds to increased membrane move-
229 ment**

230 Since removal of Rvs167 in *rvs167Δsh3* cells, and the reduced amount of Rvs167 Δ sh3 recruited in
231 *rvs167Δsh3* cells results in decreased Sla1 movement, we wondered if Sla1 movement would scale
232 with amount of Rvs recruited to endocytic sites. We titrated the amount of Rvs expressed in cells by
233 endogenously duplicating the Rvs167 and Rvs161 genes (Huber et al. 2014) in diploid and haploid
234 yeast cells (Fig.5) . We thus made diploid strains with 4x copies of both the Rvs genes (4xRVS), 2x
235 copies (WT diploid cells, 2xRVS), and 1x copy (1xRVS). Number of molecules of Rvs167 recruited
236 to endocytic sites increases with gene copy number (Fig5A). "Excess" Rvs recruited to endocytic
237 sites in the 4xRVS case does not change the rate or total movement of Sla1, or of Rvs167 (Fig.6B,C)
238 compared to the WT (2xRVS). In the case of 1xRVS, Sla1 movement is slightly reduced after 100nm
239 (Fig.6B). Magnitude of Rvs167 inward movement was similar in all three, but the Rvs167-eGFP signal
240 was lost immediately after the inward movement in the 1xRVS case, unlike in the 4xRVS and 2xRVS
241 cases, likely because fewer molecules are recruited (Fig.6A). Unlike in the *rvs167Δsh3* case, Abp1
242 and Rvs167 peaks were concomitant in all three strains, with similar amounts of Abp1 recruited
243 irrespective of Rvs gene copies (Fig.6D). Thus was there no apparent disruption of the actin network,
244 or of the coupling between scission and actin network disassembly. Adding more Rvs than in the
245 WT diploid case did not lead to differences in Sla1 movement, although reducing the amount of Rvs-
246 as in the 1xRVS case- marginally decreased movement.

247 In haploid cells, we duplicated the full-length Rvs167 gene, as well as *rvs167Δsh3* gene (Fig5E-H).
248 We thus produced strains with 2x copies of the Rvs genes (2xRVS), 1x copy of each (WT haploid,
249 1xRVS), 2x copies of the *rvs167Δsh3* gene (2xBAR), or 1 copy of *rvs167Δsh3* gene (1xBAR). Amount of
250 WT and mutant Rvs167 molecules recruited at endocytic sites varied in these strains between 50
251 and 180 copies (Fig5E). Sla1 dynamics remained the same in Rvs duplicated strain (2xRVS) as in the
252 WT (Fig.6F). In the 2xBAR case, the amount of Rvs167 Δ sh3 molecules recruited to endocytic sites
253 increased (Fig.6E), as did Sla1 movement, as well the inward jump of Rvs167 (Fig.6F,G), compared
254 to 1xBAR. Total Abp1 numbers recruited were reduced in 1xBAR (that is *rvs167Δsh3*), compared to
255 the 2xBAR, 1xRVS and 2xRVS (Fig5H). Higher Abp1 numbers corresponds to larger Sla1 centroid
256 movement in both diploid and haploid cells (Fig.6C, D, G, H), suggesting a correlation between the
257 maximum number of Abp1 recruited and total invagination length.

258 **Discussion**

259 Recruitment and function of the Rvs complex has been studied in this work, and several existing
260 models for membrane scission have been tested. We propose that Rvs is recruited to endocytic
261 sites via interactions between the Rvs BAR domains and invaginated membrane, and that SH3

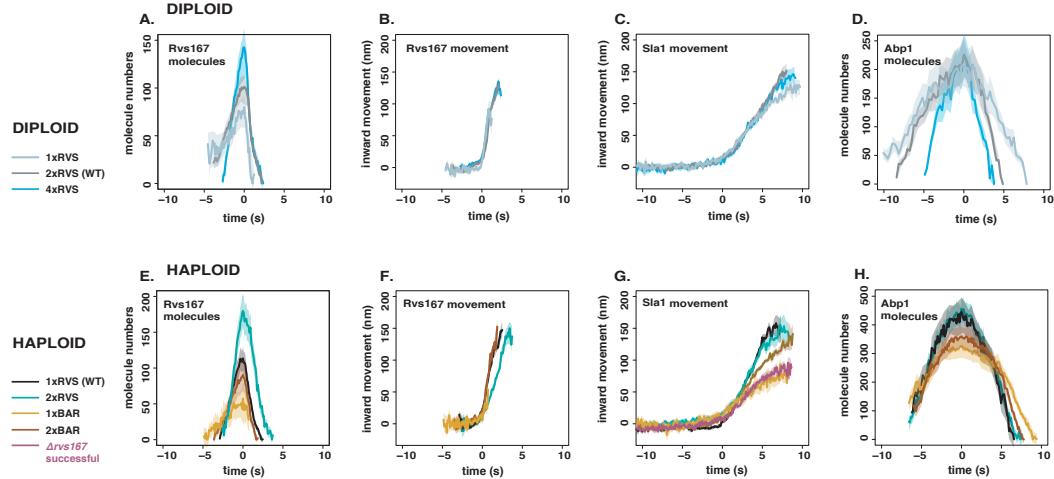


Figure 6. RVS duplication in haploid and diploid cells **A:** Recruitment of Rvs167 in diploid strains with different copy number of Rvs167 and Rvs161 genes. **B:** Rvs167 centroid positions in these strains **C:** Sla1 centroid positions in these strains. **D:** Abp1 molecule numbers in same strains, with only one Abp1 allele tagged. **E,F:** Recruitment and centroid positions of Rvs167 and Rvs167 $\Delta sh3$ in haploid strains. **G:** Sla1 centroid positions in these strains. **H:** Abp1 recruitment in the same strains. All centroid positions were aligned in the time axis so that time=0(s) corresponds to beginning of inward movement of each average centroid. Centroids were aligned in the y axis so that y=0(nm) corresponds to the beginning of the average centroid position

mediated protein-protein interactions are required for efficient recruitment of Rvs. We found that arrival of Rvs at the membrane invagination scaffolds the membrane and prevents membrane scission. WT invagination lengths depend on recruitment of a critical number of Rvs molecules. Both timing and recruitment efficiency appear crucial to Rvs function.

266 **BAR domains sense *in vivo* membrane curvature and time recruitment of Rvs**

267 The curved structure of Endophilin and Amphiphysin BAR dimers (*Peters et al., 2004; Mim et al.,*
 268 *2012*) In the absence of membrane curvature - in *sla2 Δ* cells - Rvs167 $\Delta sh3$ domains do not localize
 269 to endocytic sites (Fig.5B). This demonstrates for the first time that the BAR domain senses and
 270 requires membrane curvature to localize to endocytic sites. Rvs167 $\Delta sh3$ has a similar average
 271 lifetime at endocytic sites as full length Rvs167 (Fig.5C,D). However, time alignment with Abp1 shows
 272 that there is a delay in the recruitment of Rvs167 $\Delta sh3$, (Fig.5B). Sla1 moves inwards at a slower rate
 273 in *rvs167 $\Delta sh3$* cells, so it takes longer for the membrane in these cells to reach the same invagination
 274 length as in WT. We propose that Rvs recruitment is timed to specific membrane invagination length-
 275 therefore to a specific membrane curvature- accounting for the delay in recruitment. The timing of
 276 recruitment is therefore provided by the BAR domain.

277 **SH3 domains allow efficient and actin independent recruitment**

278 Rvs167 $\Delta sh3$ accumulates to about half the WT number (Fig.5C,D), even though the same cyto-
 279 plasmic concentration is measured (Fig.5 supplement), indicating that loss of the SH3 domain
 280 decreases the efficiency of recruitment of Rvs. In *sla2 Δ* cells, full-length Rvs167 forms patches
 281 on the membrane (Fig.3B). Since Rvs167 $\Delta sh3$ does not localize to the plasma membrane in *sla2 Δ*
 282 cells, localization of the full-length protein must be mediated by the SH3 domain. That full-length
 283 Rvs167 is able to assemble and disassemble at cortical patches in *sla2 Δ* cells without the curvature-
 284 dependent interaction of the BAR domain (Fig.3B) indicates that the SH3 domain can mediate
 285 both the recruitment and disassembly of Rvs at endocytic sites. In *sla2 Δ* cells treated with LatA
 286 (Fig.3B), both membrane curvature and actin-interacting proteins are removed from endocytic sites.
 287 Full-length Rvs167 in these cells still shows transient localizations at the plasma membrane: the

288 SH3 domain is able to localise the Rvs complex in an actin and curvature independent manner.

289 **Loss of SH3 domain disrupts endocytic actin network dynamics**

290 In WT cells, the Abp1 and Rvs167 fluorescent intensities reach maxima concomitantly (Fig.5C,D),
291 and the consequent decay of both coincide. Coincident disassembly indicates that upon vesicle
292 scission, the actin network is immediately disassembled. Membrane scission occurs around the
293 intensity peak of the two proteins (*Kukulski et al., 2012; Picco et al., 2015*). This coincident peak is
294 lost in bar-gpa cells: Rvs167Δsh3 average fluorescent intensity peaks several seconds after Abp1
295 intensity starts to drop, and the decay of Abp1 is prolonged, taking nearly double the time as in WT.
296 Although it is not clear what the decoupling of Abp1 and Rvs167Δsh3 peaks means, the changes in
297 Abp1 dynamics suggests a strong disruption of the actin network dynamics.

298 **Rvs acts as a membrane scaffold preventing membrane scission**

299 Invaginations in *rvs167Δ* cells undergo scission when the Sla1 centroid has moved about 80nm
300 (Fig.1F), compared to the WT lengths of 140nm. This shows that enough forces are generated at
301 80nm to cause scission. Since invagination lengths of *rvs167Δ* cells are increased by overexpression
302 of the Rvs167Δsh3 domains (Fig.6E-G), we think that localization of Rvs BAR domains to the
303 membrane tube stabilizes the membrane (*Boucrot et al., 2012; Dmitrieff and Nédélec, 2015*) This
304 allows the invagination to grow until actin polymerization produces enough forces to sever the
305 membrane. The requirement for Rvs scaffolding cannot be removed by reducing turgor pressure
306 (Fig.6supplement? or 7?), so the function of the scaffold is not to counter turgor pressure. There is
307 a limit to the stabilization by BAR domains: in diploid strains with 4 copies of each RVS gene, the
308 same amount of actin is recruited before scission. The invaginations lengths are the same as in the
309 other strains even though more Rvs is recruited. It is possible that the nature of the Rvs complex
310 interaction with the membrane changes after a certain amount of Rvs is recruited.

311 If enough forces are generated at 80nm, why is scission efficiency decreased in *rvs167Δ* compared
312 to WT? Forces from actin may be at a threshold when the invagination is at 80nm. There
313 could be enough force to sever the membrane, but not enough to sever reliably. The Rvs scaffold
314 then keeps the network growing to accumulate enough actin to reliably cause scission. Controlling
315 membrane tube length could also be a way for the cell to control the size of the vesicles formed,
316 and therefore the amount of cargo packed into the vesicle.

317 **What causes membrane scission?**

318 We looked for changes in the dynamics of Sla1 and Rvs167 that would indicate a scission defect
319 in various mutant strains: longer invaginations than in WT, so Sla1 centroid movements of over
320 140nm, and a bigger inwards jump of Rvs167 centroid, indicating that a longer invagination has
321 been cut. In *vps1Δ* cells, no major changes are seen in Sla1 or Rvs167 dynamics. We conclude that
322 even if Vps1 is recruited to endocytic sites, it is not necessary for Rvs localization or function, and is
323 not necessary for scission.

324 In the lipid hydrolysis model, synaptojanins hydrolyze PIP₂ molecules that are not covered by
325 BAR domains, resulting in a boundary between hydrolyzed and non-hydrolyzed PIP₂. Interfacial
326 forces generated at this lipid boundary causes scission (*Liu et al., 2006*). Deleting synaptojanins
327 Inp51 and Inp52 should increase invagination lengths if scission was driven by lipid hydrolysis.
328 Sla1 and Rvs centroid dynamics shows that deletion of neither Inp51 nor Inp52 result in scission
329 delay. In *inp51Δ* cells, Rvs assembly is slightly slower than that in WT: Inp51 could play a role in
330 Rvs recruitment. In the *inp52Δ* strain, about 12% of Sla1-GFP tracks retract, this could suggest
331 a moderate influence of Inp52 on scission. Rvs centroid persists after scission in *inp52Δ* cells:
332 disassembly of Rvs after scission is delayed. Sla1 signal also persists for longer after scission in the
333 *inp52Δ* than in WT cells, suggesting that post-scission disassembly of proteins from the vesicle is
334 inhibited in *inp52Δ* cells. Inp52 likely plays a role in recycling endocytic proteins from the vesicle to
335 the plasma membrane.

336 A protein-friction model has proposed that BAR domains induce a frictional force on the mem-
337 brane, causing scission (ref!). If more BAR domains were added to the membrane tube at a faster
338 rate, the frictional force generated as the membrane is pulled under it should increase, and the
339 membrane should rupture faster. That is, membrane scission should occur as soon as WT forces
340 are generated on the tube. In Rvs duplicated cells, adding up to 1.6x the WT amount of Rvs at faster
341 rates to membrane tubes does not affect the length at which the membrane undergoes scission
342 (Fig.6E). We think that protein friction does not contribute significantly to membrane scission in
343 yeast endocytosis.

344
345 We observed that the maximum amount of Abp1 measured in all the diploid strains is about 220
346 molecules (Fig.6D). Since only one allele of Abp1 is fluorescently tagged in these strains, the total
347 amount of Abp1 recruited is about 440 ± 20 molecules. In WT haploid cells, the maximum number
348 of Abp1 measured is 460 ± 20 molecules. We propose that recruitment of a similar amount of Abp1
349 before scission in all these strains indicates that scission is dependent on the amount of Abp1,
350 and correspondingly, on the amount of actin recruited. We propose that actin supplies the forces
351 necessary for membrane scission. The membrane invagination continues until the "right" amount
352 of actin is recruited. The amount of force necessary is determined by the physical properties of the
353 membrane like membrane rigidity, tension, and proteins accumulated on the membrane (**Dmitrieff**
354 **and Nédélec, 2015**). Vesicle scission releases membrane-bound Rvs, resulting in release of the SH3
355 along with BAR domains. Release of the SH3 domains could indicate to the actin network that
356 vesicle scission has occurred, beginning disassembly of actin components.

357 **Model for membrane scission**

358 We propose that Rvs is recruited to sites by two distinct mechanisms. SH3 domains cluster Rvs at
359 endocytic sites, increasing the efficiency with which the BAR domains sense curvature on tubular
360 membranes. BAR domains bind to endocytic sites by sensing tubular membrane. Membrane
361 shape is stabilized by BAR-membrane interaction against fluctuations that could cause scission.
362 This prevent actin forces from rupturing the membrane, and the invaginations continue to grow in
363 length as actin continues to polymerize.

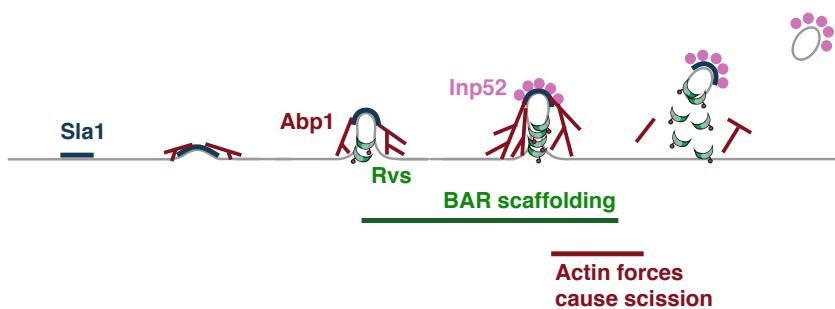


Figure 7. Model for yeast endocytic scission

364 As actin continues to polymerize, enough forces are generated to overcome the resistance to
365 membrane scission provided by the BAR scaffold. The membrane ruptures, and vesicles are formed.
366 Synaptojanins may help recruit Rvs at endocytic sites: Inp51 and Inp52 have proline rich regions
367 that could act as binding sites for Rvs167 SH3 domains. They are involved in vesicle uncoating
368 post-scission, likely by dephosphorylating PIP₂ and inducing disassembly of PIP₂-binding endocytic
369 proteins. Eventually phosphorylation regulation allows endocytic proteins to be reused at endocytic
370 sites, while the vesicle is transported elsewhere into the cell.

371 **Methods and Materials**

372 **Homologous recombination with PCR cassette insertion**

373 Tagging or deletion of endogenous genes was done by homologous integration of the product
374 of a Polymerase Chain Reaction using appropriate primers and a plasmid containing a selection
375 cassette and fluorescent tag, or only selection cassette for gene deletions. Primers were designed
376 according to Janke et al, 2004. PCRs used the Velocity Polymerase for fluorescent tagging, and Q5
377 for gene deletions using the NAT cassette. All fluorescently tagged genes have a C-terminus tag and
378 are expressed endogenously. Gene deletions and fluorescent tags are checked by PCR. Vps1del and
379 gene duplications were confirmed by sequencing.

380 **Live-cell imaging and electron microscopy**

381 Sample preparation for live imaging

382 40 μ L Concanavalin A (ConA) was incubated on a coverslip for 10 minutes. 40 μ L Yeast cells incubated
383 overnight at 25C in imaging medium SC-TRP was added to the coverslip after removing the ConA,
384 and incubated for another 10 minutes. Cells were then removed, adhered cells were washed 3x in
385 SC-TRP, and 40 μ L SC-TRP was finally added to the coverslip to prevent cells from drying.

386 Sample preparation for live imaging in LatA and sorbitol treated cells

387 Cells went through the same procedure as above till the last washing step. Instead of SC-TRP, 100x
388 diluted LatA, or Sorbitol at a final concentration of 0.2M in SC-TRP was added to the adhered cells.
389 For LatA experiments, cells were incubated in LatA for 10 minutes before imaging. For sorbitol
390 treatments, cells were imaged within 5 minutes of adding sorbitol.

391 Epifluorescent imaging for centroid tracking

392 Live-cell imaging was performed as in (*Picco et al., 2015*) Picco et al., 2015. All images were obtained
393 at room temperature using an Olympus IX81 micro- scope equipped with a 100 \times /NA 1.45 PlanApo
394 objective , with an additional 1.6x magnification lens and an EMCCD camera. The GFP channel was
395 imaged using a 470/22 nm band-pass excitation filter and a 520/35 nm band-pass emission filter.
396 mCherry epifluorescence imaging was carried out using a 556/20 nm band-pass excitation filter and
397 a 624/40 band-pass emission filter. GFP was excited using a 488 nm solid state laser and mCherry
398 was excited using a 561 nm solid state laser. Hardware was controlled using Metamorph software.
399 For single-channel images, 80-120ms was used as exposure time. All dual-channel images were
400 acquired using 250ms exposure time. Si- multaneous dual-color images were obtained using a
401 dichroic mirror, with TetraSpeck beads used to correct for chromatic abberation.

402 Epifluorescent imaging for molecule number quantification

403 Images were acquired as in Picco et al., 2015. Z-stacks of cells containing the GFP-tagged protein
404 of interest, incubated along with cells containing Nuf2-GFP, were acquired using 400ms exposure
405 using a mercury vapour lamp, on a CCD camera. Z stacks were spaced at 200nm.

406 **Live-cell image analysis**

407 Images were processed for background noise using a rolling ball radius of 90 pixels. Particle
408 detection, and tracking was performed for a particle size of 6 pixels, using scripts that com-
409 bine background subtraction with Particle Tracker and Detector, that can be found on ImageJ
410 (<http://imagej.nih.gov>). Further analysis for centroid averaging, alignments between dual-color
411 images and single channel images, for alignment to the reference Abp1 were done using scripts
412 written in Matlab (Mathworks) and R (www.r-project.org), written originally by Andrea Picco, and
413 modified by me. Details of analysis can be found at (*Picco et al., 2015*). All movement and intensity
414 plots from centroid tracking show the average centroid with 95% confidence interval. All molecule
415 number quantifications report either the median or maximum number of molecules with standard
416 error of mean. Maximum number is preferred over median in cases when the rate of change of

417 fluorescent intensity of two populations being compared are not similar, and the lifetime of the
418 protein populations being compared are not similar. The median in this case underreports the
419 differences in protein accumulation.

420 **Cytoplasmic background quantification**

421 On a maximum intensity projection of time-lapse images, the average pixel intensity within a circle
422 of set radius in the cytoplasm was measured. This circle is manually arranged so that cortical
423 patches were excluded, and mean intensity was acquired for about 10 cells of each cell type. A
424 fixed area outside the cells was drawn, and mean intensity was calculated to establish "background
425 intensity". This background intensity was then subtracted from the mean intensity to obtain a
426 rough measure of cytoplasmic intensity. There are some caveats with this quantification: the cells
427 were not incubated in the same field of view, cellular autofluorescence is assumed to be equal for
428 the different strains.

429 **Citations**

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438 **Acknowledgments**

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440 the acknowledgments section.

441 **References**

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562
563

564 **Supplementary Material**

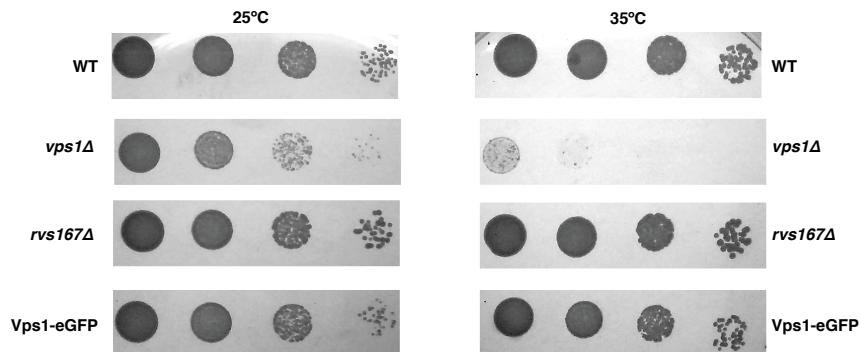


Figure S1. Growth assay of WT, *vps1Δ*, *rvs167Δ*, and cells expressing Vps1-eGFP at 25°C and 35°C

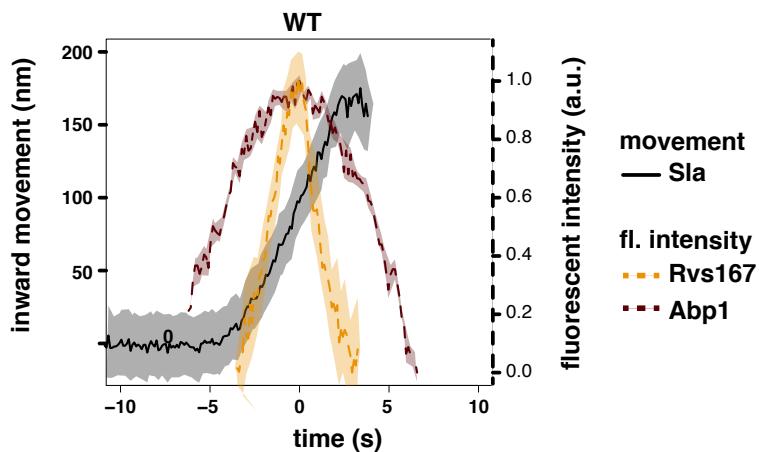


Figure S2. Sla1 centroid aligned so that time=0(s) is the maximum of Abp1 fluroescent intensity in WT cells (Picco et al., 2015), normalized Abp1 and Rvs167 fluorescent intensities in WT cells

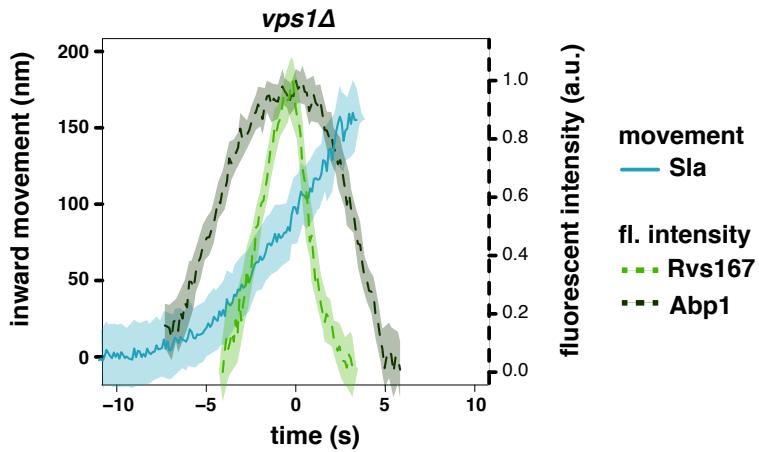


Figure S3. Sla1 centroid aligned so that time=0(s) is the maximum of Abp1 fluorescent intensity in *vps1Δ* cells (Picco et al., 2015), normalized Abp1 and Rvs167 fluorescent intensities in *vps1Δ* cells

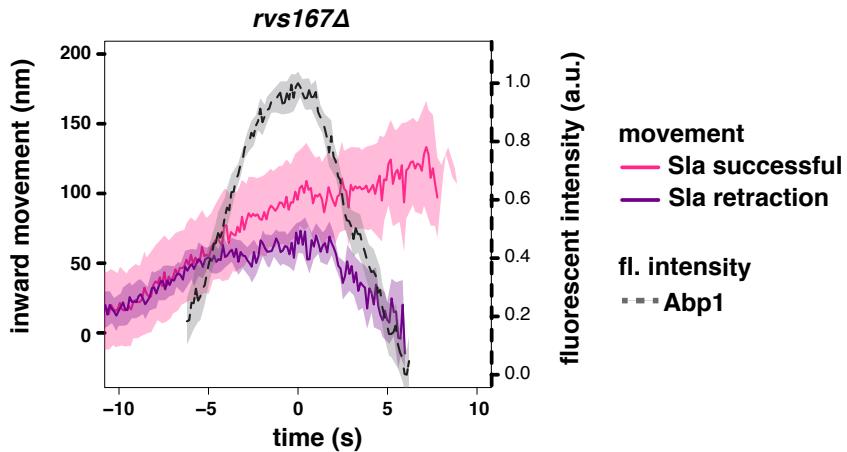


Figure S4. Sla1 centroid aligned so that time=0(s) is the maximum of Abp1 fluorescent intensity in *rvs167Δ* cells (Picco et al., 2015), normalized Abp1 fluorescent intensity in *rvs167Δ* cells

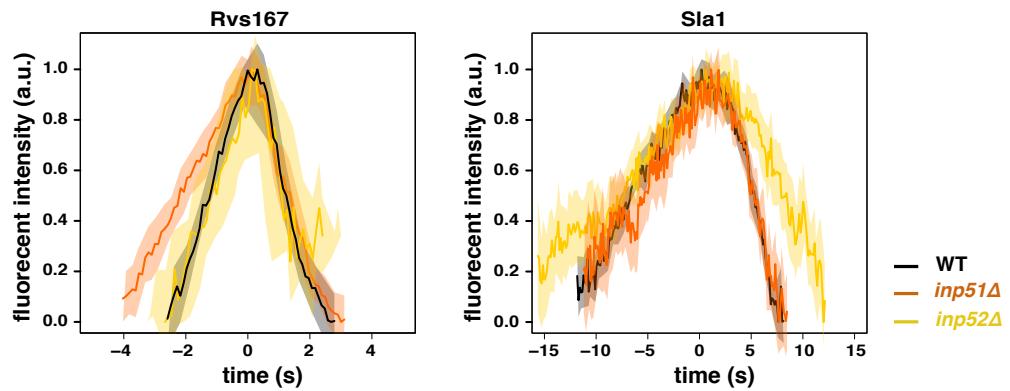


Figure S5. Normalized Rvs167 and Sla1 fluorescent intensities aligned in time so that time=0(s) is the maximum of each corresponding fluorescent intensity profile

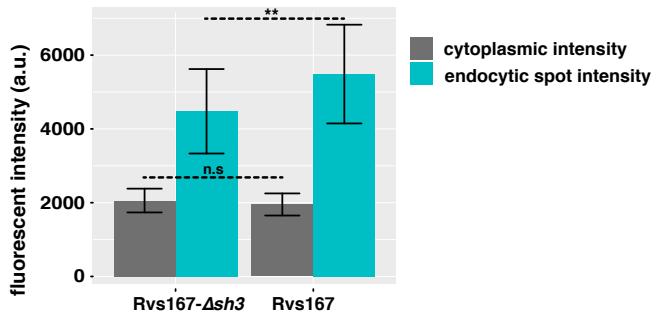


Figure S6. Cytoplasmic intensity and intensity of endocytic patches of Rvs167 and Rvs167 $\Delta sh3$ in WT and rvs167 $\Delta sh3$ cells. Error bars are standard deviation, p values from t-test, *= p ≤ 0.05, **= p ≤ 0.01, ***=p≤0.001.