Regulation of membrane scission in yeast endocytosis

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Abstract

Introduction

In Clathrin-mediated endocytosis, a flat plasma membrane is pulled into a tubular invagination that eventually forms a vesicle. Forces that drive the transition from invagination to spherical vesicle in mammalian cells are provided by constriction of the GTPase Dynamin. Dynamin is now known to act in concert with the crescent-shaped N-BAR proteins Endophilin and Amphyphysin (ref. Dynamin papers). Proline-rich motifs on the Dynamin. In yeast cells, what causes membrane scission is unclear, although the yeast N-BAR protein complex Rvs has been identified as an important component of the scission module. In yeast, the Amphiphysin and Endophilin homologue Rvs is a heterodimeric complex composed of Rvs161 and Rvs167 (Friesen et al., 2006). Deletion of Rvs reduces scission efficiency by nearly 30% and reduces the invagination lengths at which scission occurs (ref Marko, wanda). Apart from a canonical N-BAR domain which forms a cresent-shaped structure, Rvs167 has a Glycine-Proline-Alanine rich (GPA) region and a C-terminal SH3 domain. Rvs161 and Rvs167 N-BAR domains are 42% similar, and 21% identical, but are not interchangeable (Sivadon, Crouzet and Aigle, 1997). The GPA region is thought to act as a linker with no known other function, while loss of the SH3 domain affects budding pattern and actin morphology. Most Rvs deletion phenotypes can however, be rescued by expression of the BAR domain alone (Sivadon, Crouzet and Aigle, 1997), suggesting that the BAR domains are the main functional unit of the Rvs complex. Homology modelling has shown that the BAR domain of Rvs167 is similar to Amphiphysin and Endophilin (Youn et al., 2010), and is therefore likely to function similarly to the mammalian homologues. In keeping with this theory, Rvs has been shown to tubulate liposomes in vitro (Youn et al., 2010). The Rvs complex arrives at endocytic sites in the last stage of the endocytosis, and disassembles rapidly at the time of membrane scission (Picco et al., 2015), consistent with a role in membrane scission. While it is known to be involved in the last stages of endocytosis, a mechanistic understanding of the influence of Rvs on scission however, remains incomplete. u89

We used quantitative live-cell imaging and genetic manipulation in S.cereviciae to investigate the influence of Rvs and several Rvs interacting proteins that have been suggested to have a role in scission. We found that arrival of Rvs to endocytic sites is timed by interaction of its BAR domain with a specific membrane curvature. The Rvs167 SH3 domain affects localization efficiency of the Rvs complex and also influences invagination dynamics. This indicates that both BAR and SH3 domains are important for the role of Rvs as a regulator of scission. We tested current models of membrane scission, and find that deleting yeast synaptojanins or dynamin does not change scission dynamics. Interfacial forces at lipid boundaries are therefore unlikely to be sufficient for scission, and forces exerted by dynamin are not required. Furthermore, invagination length is insensitive to overexpression of Rvs, suggesting that the recently proposed mechanism of BAR-induced protein friction on the membrane is not likely to drive scission. We propose that recruitment of Rvs BAR

domains prevents scission and allows invaginations to grow by stabilizing them. We also propose that vesicle formation is dependent on forces exerted by a different module of the endocytic pathway, the actin network. Preventing premature membrane scission via BAR interaction could allow invaginations to grow to a particular length and accumulate enough forces within the actin network to reliably cut the membrane.

Results

Rvs167 deletion results in reduced coat movement

Deletion of the Rvs genes have resulted in the scission-specific phenotype of membrane retraction: inward movement and consequent retraction of the invaginated membrane back to the cell wall (ref.Marko). Since Rvs161 and Rvs167 form dimers (ref.Dominik), deletion of Rvs167 effectively removes both proteins from endocytic sites. We therefore quantified the effect of deletion of Rvs167 on membrane invagination. To follow the membrane invagination we used Sla1, an endocytic coat protein. Upon actin polymerization, the endocytic coat is pulled along with the membrane as it invaginates (ref.Skruzny?), and thus acts as a proxy for the behaviour of the plasma membrane. We endogenously tagged Sla1 at the N-terminus with eGFP in WT and *rvs167*Δ cells (Fig.1a), and tracked the dynamics of Sla1.

27% of Sla1 patches that begin to form invaginations move inward and then retract in *rvs167*Δ cells (Fig.1d), consistent with retraction rates measured in other experiments (Kaksonen, Toret and Drubin, 2005), and suggesting failed scission in 27% of endocytic events. Movement of the retractions and of successful endocytic events were quantified (Fig.1e) as described in Picco et. al, 2015. In brief, yeast cells expressing fluorescently-tagged endocytic proteins are imaged at the equatorial plane. Since membrane invagination progresses perpendicularly to the plane of the plasma membrane, proteins that move into the cytoplasm during invagination do so in the imaging plane. Centroids of Sla1 patches- each patch being an endocytic site- are tracked in time. Between 40-50 Sla1 centroids are averaged. This provides an averaged centroid that can be followed with high spatial and temporal resolution. When different endocytic proteins are simultaneously imaged with Actin Binding Protein Abp1, Abp1 provides a frame of reference to which all the other proteins can be aligned. Abp1 is used because it is abundant at endocytic sites and therefore easily imaged. Time=0 is established as the peak of the Abp1 fluorescence intensity in respective co-tagged strains strains (Fig.1, supplement). Abp1 fluorescent intensity maxima in wild-type cells is concomitant with the peak of Rvs167 fluorescent intensity and is time window in which scission occurs (ref2andrea, refwanda).

In WT cells, the averaged Sla1 centroid moves inward at a linear rate to a distance of 140nm from its starting position. After this, the centroid position shows increased noise, likely from random motion of the vesicle post-scission. Following scission, Sla1 molecules are disassembled from the endocytic vesicle (Fig1 supplement). Sla1 centroid movement in both successful and retracting endocytic events in *rvs167*Δ cells and WT look similar up to about 60nm (Fig.1e). Consequent movement in successful scission events slows dramatically, and invaginations appear to undergo scission between 60 -80 nm. CLEM has shown that Rvs167 localizes to endocytic sites after the invaginations are about 60nm long (Kukulski et al., 2012). Sla1 movement in *rvs167*Δ shows therefore that membrane invagination is unaffected till Rvs is supposed to arrive. Sla1 in *rvs167*Δ then continues to move at a much slower rate to about 80nm. That membrane scission occurs at shorter invagination lengths than in WT is corroborated by the smaller vesicles formed in *rvs167*Δ cells (Kukulski et al., 2012). This indicates that first, membrane scission can occur at invagination lengths of 80nm. Then, that the arrival of Rvs prevents membrane scission at 80nm and allows further membrane invagination. In retraction events, after inward movement, the Sla1 centroid moves back towards the starting position, that is, to the plasma membrane.

Vps1 does not influence coat or scission dynamics

Endocytic membrane scission in mammalian cells is understood to be driven by constriction of the tubule neck by the Gtpase Dynamin (Grigliatti et al., 1973; Poodry and Edgar, 1979; van der Bliek and Meyerowrtz, 1991). Mammalian Dynamin is recruited to endocytic sites via their proline-rich domains (PRD) to SH3 domains of N-BAR proteins amphiphysin and endophilin (Grabs et al., 1997; Cestra et al., 1999; Farsad et al., 2001; Meinecke et al., 2013, Ferguson, 2009). In yeast, the Dynamin-like protein Vps1 is essential for vacuolar protein sorting, and does not contain a PRD. It is however, reportedly recruited to endocytic sites and interacts with endocytic proteins (refAyscough, Yu, 2004; Nannapaneni et al., 2010; Goud Gadila et al., 2017). Vps1 tagged both N- and C-terminally with GFP constructs failed to co-localize with endocytic proteins in our hands (Fig.1 supplement), indicating that Vps1 may not play a role in yeast endocytosis. To test whether absence of Vps1 influences scission, endocytic dynamics are observed in cells lacking Vps1 and compared against WT cells. In Fig.1d, retraction of Sla1 in *vps1*Δ and wild-type cells is quantified, suggesting that retraction rates do not increase in the absence of Vps1.

The averaged centroid trajectory of Sla1-eGFP is tracked in 50 endocytic sites in *vps1*Δ and WT cells (Fig.1e). Centroid movement of Sla1-eGFP in WT cells shows a linear movement to about 140nm. Sla1 movement in *vps1*Δ cells has the same magnitude of inward movement to about 140nm. In spite of slight differences in the rates of inward movment, that the total inward movment does not change indicates that plasma membrane dynamics remain uninfluenced by the absence of Vps1.

Centroid tracking has shown that the number of molecules of Rvs167 peaks at the time of scission, and is followed by a rapid loss of fluorescent intensity, simultaneous with a sharp jump of the centroid into the cytoplasm (ref.Andrea). This jump, also seen in Rvs167-GFP kymographs (Fig.1c), is interpreted as loss of protein on the membrane tube, causing an apparent spatial jump to the protein localized at the base of the newly formed vesicle. Kymographs of Rvs167-GFP (Fig.1c), as well as Rvs167 centroid tracking (Fig.1e) in Vps1 deleted cells show the same jump, indicating that vesicles are formed in the same position in Vps1 deletion cells as in WT cells. From the absence of change in coat as well as scission dynamics in Vps1 deleted cells, we conclude that Vps1 does not influence the endocytic process.

Synaptojanins likely influence vesicle uncoating, but not scission dynamics.

In mammalian cells, disruption of Synaptojanin genes results in cellular accumulation of PIP2 at endocytic sites. Coated vesicles gather at the plasma membrane, suggesting a role for lipid hydrolysis in releasing coat proteins from nascent vesicles (ref?). As an alternate to forces from Dynamin constriction, Liu et al (refliu) have proposed that an interaction between PiP2-hyrdolyzing Synaptojanins and BAR proteins could drive membrane scission. In this model Rvs BAR domains would form a scaffold on the membrane tube, preventing hydrolysis of underlying PIP2. Synaptojanin would arrive at inavaginated membranes, and hydrolyse unprotected PIP2. This generates a lipid boundary between BAR-protected PIP2 at the tube and hydrolyzed PIP2 at the bud tip. A line tension thus formed at the interphase between the two lipid types would then generate enough force to pinch off a vesicle.

There are three Synaptojanin-like proteins in yeast- Inp51, Inp52 and Inp53. Inp51 exhibits a diffuse cytoplasmic signal, and Inp53 localizes to patches within the cytoplasm- cellular localization that is consistent with involvement in trans-Golgi signalling (refGolgi). Inp53 was not investigated further. Inp52 localizes to cortical actin patches that are endocytic sites. Two channel alignment shows that Inp52 patches arrive in the late scission stage, and localizes to the bud tip, consistent with a role in membrane scission.

Role of Inp51 and Inp52 are tested by following Sla1-GFP and Rvs167-GFP in cells with either Inp51, Inp52, or both deleted. Retraction events do not significantly increase compared to the WT in either inp51del or inp52del cells. Magnitude and speed of coat movement in inp51del is the same as the WT. In inp52del cells, coat movement also has the same magnitude and speed, but GFP signal

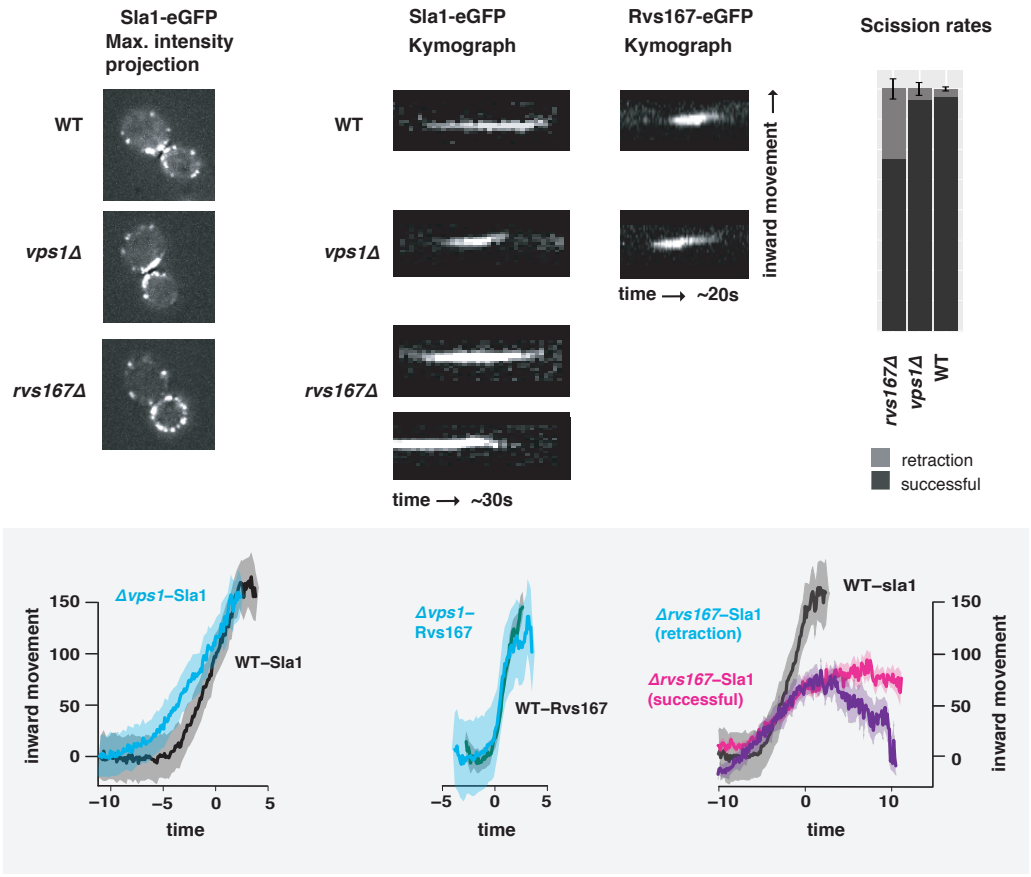


Figure 1. A half-columnwidth image using wrapfigure, to be used sparingly. Note that using a wrap figure before a sectional heading, near other floats or page boundaries is not recommended, as it may cause interesting layout issues. Use the optional argument to wrapfigure to control how many lines of text should be set half-width alongside it.

is persistant after membrane scission. This delay in decrease of Sla1-GFP signal is consistent with delay in vesicle uncoating rather than membrane scissison. Similarly, Rvs167 disassembly has a delay, while the assembly is similar to WT, indicating a delay in removing endocytic proteins from the newly formed vesicle. Assembly of Rvs167 has a delay in inp51deleted cells, which could indicate a defect in recruiting proteins to endocytic sites, or in progression of endocytic invaginations. Since Sla1 movement is the same, we suggest a defect in the former rather than latter.

Figure 2. A half-columnwidth image using wrapfigure, to be used sparingly. Note that using a wrapfigure before a sectional heading, near other floats or page boundaries is not recommended, as it may cause interesting layout issues. Use the optional argument to wrapfigure to control how many lines of text should be set half-width alongside it.

Rvs BAR domains recognize membrane curvature in-vivo

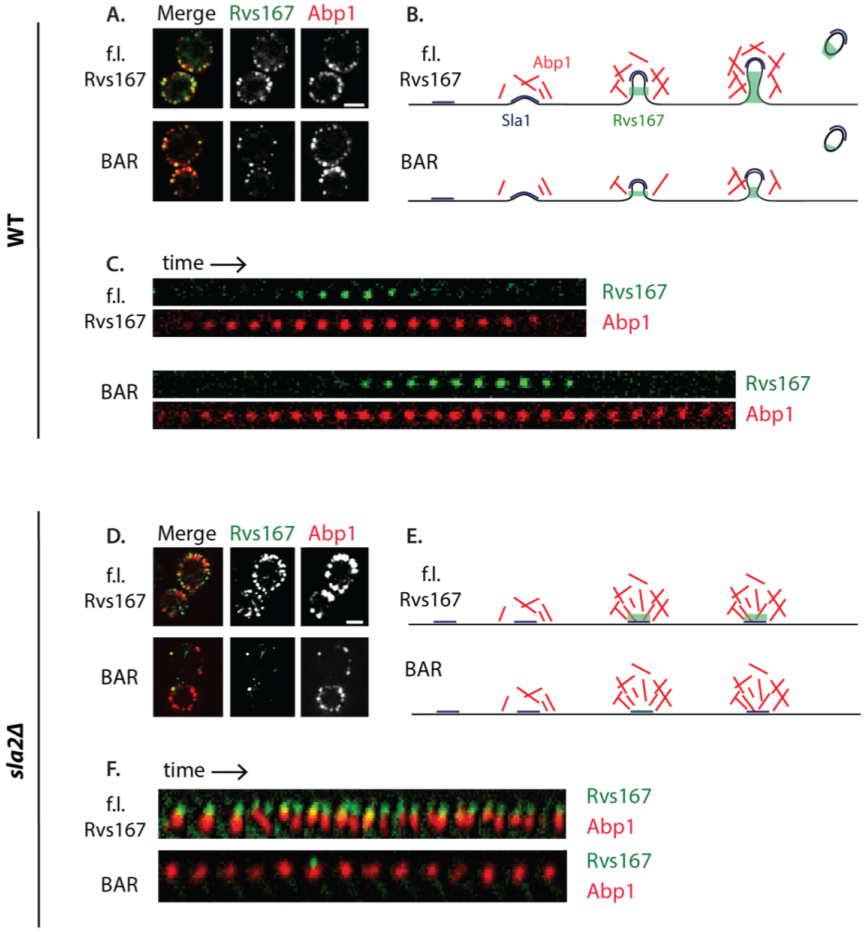
The curved tertiary structure and liposome binding assays of N-BAR domains have suggested that they may have a preference for curved membrane that match their own intrinsic curvature. Alternately, they may also impose their curvature on flat membrane and induce curvature formation. The curvature interaction of Rvs167 in vivo has not been tested. In order to do so, we deleted the SH3 domain of Rvs167 (henceforth BAR-GPA) and observed the localization of Rvs167 with and witout the SH3 domain. The GPA region is a disordered region that has no previously reported function and was retained to ensure proper folding and function of the BAR domain. Endogenously tagged Rvs167-eGFP and BAR-GPA-eGFP and Abp1-mCherry in WT and sla2deletion cells are compared. Sla2 acts as the molecular linker between forces exerted by the actin network and the plasma membrane (ref. Skruzny). Sla2deletion cells therefore contain polymerizing actin network at endocytic patches, but the membrane remains flat and endocytosis fails. In these cells, the full-length Rvs167 protein co-localizes with Abp1-mCherry, indicating that it is recruited to endocytic sites. BAR-GPA-eGFP localization is removed, except for rare transient patches that do not co-localize with Abp1-mCherry, indicating that in the absence of membrane curvature, the BAR domains cannot localize to endocytic sites.

Rvs SH3 domains contribute to curvature independent localization

We have shown that BAR domains need membrane curvature to localize. Full-length Rvs167, however, is recruited to endocytic patches in sla2deletion cells. This indicates that a second interaction- that is not the BAR-curvature dependent- recruits the protein to endocytic sites. This interaction must come from the SH3 region, showing that Rvs localization is dependent on both BAR as well as SH3 domain interactions. Absence of the SH3 domain also reduces total recruitment of Rvs and Abp1 protein, giving the SH3 domain an important and surprising role in regulating the late stage of endocytosis.

SH3 domains are recruited by Myosin 5

SH3 domains have been shown to interact with several proteins in the actin module of endocytosis: Las17, type I myosins, and Vrp1 all have genetic or physical interactions with Rvs167 SH3 domains (Lila and Drubin, 1997; Colwill et al., 1999, Madania et al., 1999; Liu et al., 2009). We tested the interaction by studying the localization of full-length Rvs167 in cells with one of these proteins deleted, and treated with LatA to reproduce the situation in which BAR-curvature interaction is removed. Deletion of neither Las17 nor Myo3 in combination with LatA treatment does not remove the localization of Rvs167. Deletion of Vrp1 and Myo5, with LatA treatment removes localization of Rvs167. Since Vrp1 is required for the recruitment of Myo5 (refMyo5), SH3 domains likely interact with Myo5 rather than Vrp1.



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what about the differences in myo5 and myo3 number... if the Rvs recruitment only slightly depended on myo3 we probably wouldnt see a difference

N-helix and GPA domains do not contribute to recruitment of Rvs or membrane movement

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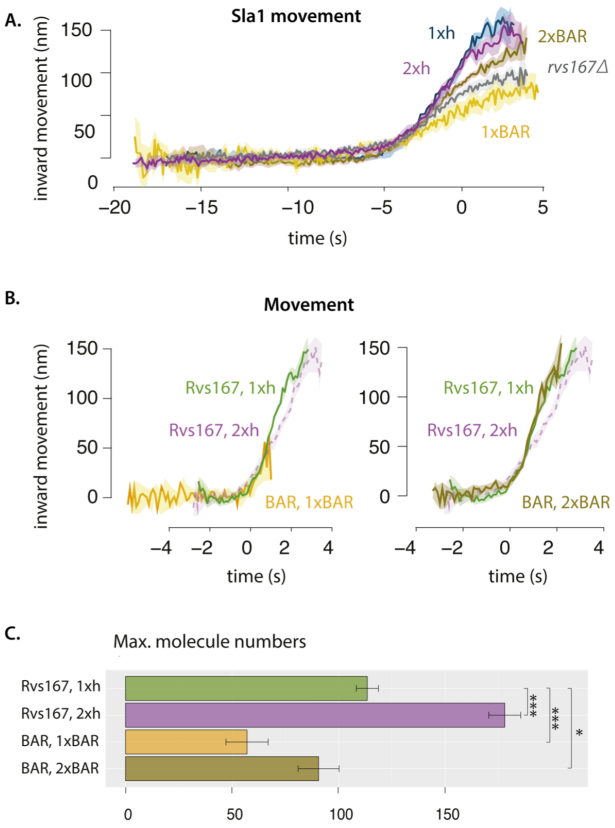
Increased BAR domain recruitment corresponds to increased membrane movement

The decreased Sla1 movement in BAR cells can be explained by a loss of interaction of the SH3 domain, or by reduced recruitment of the BAR domains. To check whether increasing the recruitment of the Rvs complex alone can rescue reduced Sla1 movement, the Rvs167 and Rvs161 ORF

was duplicated endogenously (ref Huber dude) in diploid and haploid yeast cells. In diploid cells, Rvs duplication results in either 4x copies of both Rvs genes, 2x copies (WT diploid) or 1x copies, in which one gene of Rvs167 and Rvs161 are deleted. We show that amount of Rvs167 recruited to sites increases linearly, without changing either the rate of movement or total movement before scission of Sla1. Similarly, in haploid cells, increasing the gene copy of Rvs167 and Rvs161 results in increased recruitment of Rvs167, without influencing the dynamics of Sla1. Expressing two instead of one copy of the Rvs167 BAR domain alone rescues the loss of Sla1 movement in the 1x copy of BAR domain alone, as well the inward jump of BAR-GFP itself. The loss of inward movement in 1xBAR suggests that smaller vesicles are produced in these cells, confirmed by CLEM. This would in corollary indicate that the increased inward movement in 2xBAR produces WT-sized vesicles.



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Discussion

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Methods and Materials

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